STRESS AND IMMUNE RESPONSES OF RANCHED SOUTHERN BLUEFIN TUNA (*THUNNUS MACCOYII* CASTLENEAU).

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Doctor of Philosophy

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DEDICATION

To my wife, Ruth, and sons, Matt and Sam
for their support and encouragement over the years.
To my mother, Pauline, and late father, Frank
for their sacrifices to provide my brothers and I
the educational opportunities denied them.

It took a while ‘Frankie’ but we did it.

DECLARATIONS AND STATEMENTS

Declaration of Originality

This thesis contains no material which has been accepted for a degree or diploma by the University or any other institution, except by way of background information and duly acknowledged in the thesis, and to the best of my knowledge and belief no material previously published or written by another person except where due acknowledgement is made in the text of the thesis, nor does the thesis contain any material that infringes copyright.

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Statement of Ethical Conduct

The research associated with this thesis abides by the international and Australian codes on human and animal experimentation, the guidelines by the Australian Government's Office of the Gene Technology Regulator and the rulings of the Safety, Ethics and Institutional Biosafety Committees of the University. The research methodology received clearance from the University of Tasmania Experimentation Ethics Review Committee (Approval number A0008941).

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ABSTRACT

Aquatic organisms under culture conditions are faced with an assortment of potential stressors that challenge the homeostatic state. The stress and immune responses of ranched southern bluefin tuna (SBT), *Thunnus maccoyii* were investigated. Quota restrictions by the CCSBT progressively had reduced Australia's allocation of the SBT global TAC to 5,265 tonnes per annum for the period 1989 to 2009. In response to quota reductions, SBT ranching developed as a capture-based aquaculture operation dependent on wild sourced stock. Other capture-based aquaculture operations exist in Japan and Mexico for Pacific bluefin tuna (PBT), *Thunnus orientalis*, and in numerous Mediterranean countries for Atlantic bluefin tuna (ABT), *Thunnus thynnus*. Overseas operations initially used traditional catch methods, like set net traps and line hooking, to source wild bluefin, but in the mid 1990s adopted the SBT model of purse seine capture and tow cage transport to grow-out sites. SBT capture/transport mortalities (0.5% down to 0.08% in recent years) compared favourably to overseas experience (ABT up to 2%, PBT 1 to 3%) as did initial total grow-out mortalities (2 to 5% for SBT, 3 to 5% for ABT, 6% for PBT). Increasing SBT mortalities in the mid 2000s, with an annual spike from 6 to 11 weeks after transfer, have been attributed to the aporocotylid parasites *Cardicola forsteri* and *C. orientalis*.

Following an analysis of industry tow practices, suggested optimal parameters for SBT operations were tow densities of less than 0.3 fish / m^3, tow biomass less than 5 kg / m^3 and average tow speeds of around 0.5 knots. Stress indicators including cortisol, glucose, lactate, pH, osmolality, haemoglobin, insulin-like growth factor (IGF) and immune responses lysozyme and immunoglobulin (IgM) were of varying utility in elucidating SBT health aspects under ranching conditions. Suggested base reference levels in wild SBT were cortisol 10 to 70 ng/mL, glucose 5 to 10 mmol/L, lactate 1 to 10 mmol/L, pH 7.5 to 8.5, osmolality 375 to 460 mOsmol/kg, haemoglobin 13 to 21 g/dL, IGF 12 to 55 ng/mL, lysozyme 5 to 40 µg/mL and IgM 4 to 16 mg/mL. An acute primary stress response was evident at the completion of tows and elevated cortisol, lactate, osmolality and lysozyme levels suggested a modest chronic stress response throughout the ranching period. IGF levels mid season suggested production potential was not influenced greatly by chronic stress. Glucose, pH and IgM levels were not affected over ranching timeframes. Cortisol, lactate and osmolality levels under long-term holding (LTH) of SBT indicated the stress response was heightened and endured over the 18 month grow-out period. LTH glucose levels also indicated a stress response although lysozyme levels were less responsive within the 2005 LTH SBT cohort.

SBT appear to have a greater surface area cover of melanomacrophages (MMCs) within their haemopoietic organs than many fish. Kidney MMC area (1.21 $\pm$ 0.17 %) was unresponsive to the influences of ranching whereas splenic MMC area (1.25 $\pm$ 0.13 %) increased in a significant and predictable manner up to 2.36 times the initial area. Liver MMCs were very seldom detected in ranched SBT. The pigments melanin, haemosiderin and lipofuscin were all present in SBT MMCs, with kidney MMCs having more lipofuscin than haemosiderin, and splenic MMCs having high haemosiderin and less melanin. It was suggested that the increased splenic deposition likely was due to an increased exposure to exogenous waste material associated with infection by parasites, *Cardicola* spp.

This study recommended optimal tow practices for SBT capture-based operations and documented stress and immune responses as a result of tows, and of ranching over grow-out periods of 6 to 8 months. It provided collaborating evidence for the extent and timing of the onset of substantive adverse impacts from the infection of ranched SBT by *Cardicola* spp., around 2008. It documented the heightened and enduring stress response of SBT under an extended grow-out period and the associated immunosuppression from reduced complement activity pathways. The MMC characteristics of the kidney and spleen of SBT were documented for the first time and suggested a high capacity to process antigens and other foreign material to which aquaculture species can be exposed. Splenic MMCs were suggested to be a reliable, post-hoc measure for comparison of SBT health performance under ranching conditions.

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A special thanks to my supervisor Professor Barbara Nowak for her wise counsel and creative suggestions, and particularly for the compassionate yet firm 'hand' that was required to get an, if not wayward, at least somewhat distracted post graduate student over the line.

Thank you Barbara.

LIST OF ABBREVIATIONS

°C	Degrees centigrade
ABT	Atlantic bluefin tuna (*Thunnus thynnus*)
ACH	Alternative Complement Haemolysis
ACH50	Alternative Complement Haemolysis activity for 50% lysis
ACTH	Adrenocorticotropic Hormone
AFMA	Australian Fisheries Management Authority
ANOVA	Analysis of Variance
Ant.	Antartic
Aquafin CRC	Aquafin Cooperative Research Centre for Sustainable Aquaculture
ASBTIA	Australian Southern Bluefin Tuna Industry Association
CCSBT	Commission for the Conservation of Southern Bluefin Tuna
CH	Complement Haemolysis
CH50	Complement Haemolysis activity for 50% lysis
Cort	Cortisol
Cox2	Cyclooxygenase-2
CRF	Corticotropin-releasing factor
Dens	Density
DHA	Docosahexaenoic acid
DNA	Deoxyribonucleic acid
E	Extreme
ELISA	Enzyme-linked immunosorbent assay
EPA	Eicosapentaenoic acid
FAO	Fisheries and Agricultural Organisation of the United Nations
FCR	Food conversion ratio
FRDC	Fisheries Research and Development Corporation
GAB	Great Australian Bight
g/dL	Grams per decalitre
g/L	Grams per litre
Gluc	Glucose
GM	Genetically modified
H	High
H&E	Haematoxylin and eosin
h	Hour
Hb	Haemoglobin
HBSS	Hanks balanced salt solution
HSD	Honest Significant Difference
Hsp70	Heat shock proteins of 70 kilodaltons molecular mass
IATTC	Inter-American Atlantic & Tropical Tuna Commission
ICCAT	International Commission for the Conservation of Atlantic Tunas
Ig(M)	Immunoglobulin (M)
IGF(I)	Insulin-like growth factor (I)
IL-1β	Interleukin-1 beta
IL-8	Interleukin-8
J	Juvenile
K	Kidney
K values	Measure of muscle nucleotide breakdown
kg(s)	Kilogram(s)
kg/m^3	Kilograms per cubic metre
kms	Kilometres
kts	Knots

LIST OF ABBREVIATIONS (CONTINUED)

L	Liver
Lac	Lactate
Lat	Latitude
LMF	Lincoln Marine Farm
LMSC	Lincoln Marine Science Centre
Long	Longitude
LPS	Lipopolysaccharides
LTH	Long term holding
Lw	Low
Lys	Lysozyme
m	Metre
M	Medium
m^3	Cubic metres
Max	Maximum
mg	Milligram
mg/dL	Milligram per decalitre
mg/mL	Milligram per millilitre
mg O_2/kg/h	Milligrams of oxygen per kilogram per hour
Mid seas	Middle of season
min	Minutes
Min	Minimum
mm^2	Square millimetres
MMCs	Melanomacrophage Centres
mmol/kg	Millimole per kilogram
mmol/mL	Millimole per millilitre
morts	Mortalities
mOsmol	MilliOsmol
MS_{error}	Mean Square Error
MSH	Melanocyte stimulating hormone
$n_{(i)}$	Sample size$_{(i\ th\ sample)}$
NA	Not available
NaCl	Sodium chloride
Na_2PO_4	Sodium phosphate dibasic
NIR	Near Infrared Spectroscopy
ng/mL	Nanograms per millilitre
nm	Nautical miles
nmol/L	Nanomole per litre
No	Number
NS	No sample available
NSW DPI	New South Wales Department of Primary Industry
Osmo	Osmolality
P	Probability
PBT	Pacific bluefin tuna (*Thunnus orientalis*)
PCB	Polychlorinated biphenyl
PCDD/F	Polychlorinated dibenzo-p-dioxin and dibenzofuran
PCR	Polymerase Chain Reaction
qPCR	Quantitative Polymerase Chain Reaction
R&D	Research and Develpoment
R^2	Coefficient of determination
REPRO-DOTT	Reproduction for the domestication of *Thunnus thynnus*
RMR	Routine Metabolic Rate

LIST OF ABBREVIATIONS (CONTINUED)

RIA	Radioimmunoassay
r_s	Spearman's rho correlation coefficient
S	Spleen
$s^2_{(i)}$	Variance of sample group *i*
SAMF	South Australian Marine Farm
SBT	Southern bluefin tuna (*Thunnus maccoyii*)
SELFDOTT	Self sustained aquaculture and domestication of *Thunnus thynnus*
SMR	Standard Metabolic Rate
SPSS	Statistical Package for Social Sciences
SRBC	Sheep red blood cells
SST	Sea surface temperature
SYBR Green	Proprietary dye for staining nucleic acid
T	Temperature
TAC	Total Allowable Catch
TBARS	Thiobarbituric acid reactive substances
TNF-α	Tumour necrosis factor-α
T-C Ratios	Trypsin:Chymotrypsin ratios
ug HEWL/mL	Micrograms Hen Egg White Lysozyme per millilitre
ug/mL	Micrograms per millilitre
um	Micrometres
units/mL	Units per millilitre
US	United States
YTK	Yellow tail kingfish (*Seriola lalandi*)

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CHAPTER 1 INTRODUCTION

Life kills. Indeed a good part of life's resource is spent in avoiding the alternative. The challenge for the fish farmer is to get a product to market before life succeeds. Fortunately, life also seeks the status quo, the maintenance of a stable bodily environment within which an organism's principle pursuits of growth and reproduction can ensue. Challenges to the homeostatic state represent stress and aquatic organisms are faced with an assortment of potential stressors. This largely is a result of the intimate contact required with the surrounding aqueous environment in order to move and undertake the exchange of gases, the intake of nutriment and the excretion of wastes. Perhaps intuitively it would seem that a pelagic, high order predatory fish that is an obligate ram ventilator such as the southern bluefin tuna (SBT), *Thunnus maccoyii*, might encounter substantive homeostatic challenges under the confinement of the transport and grow-out regimes required for culture. Note nomenclature for the *Thunnus* genus follows that of Collette et al (2001). This study investigated the capture/transport and grow-out of ranched SBT and some of their associated stress and immune responses.

1.1 TUNA PHYSIOLOGY

Tuna have many morphologic and physiologic distinguishing features that enable them to maintain an active pelagic existence. Relative to most other fish species tuna display a unique swimming action, increased physiological processes including metabolic rate, aerobic and cardiac function, and endothermic ability. A brief synopsis is given here to provide a physiological context for subsequent discussions on stress and ranching. Comprehensive reviews are provided by Block and Stevens (2001) and Graham and Dickson (2004). Tuna employ a thunniform swimming action characterised by minimal lateral sideways movement of the body and a powerful stroke of its lunate tail. They have a small swim bladder and require constant and relatively rapid swimming to generate lift to prevent sinking. They are unable to use operculum contractions to pump water over their gills as do most fish, and must rely on ram ventilation of water across the gill surface as they move through the water. Commensurate with the constant swimming requirement is a higher rate of aerobic metabolism. Standard metabolic rate (SMR) is the resting and fasting metabolism at a given temperature and is the theoretical minimum metabolic rate. For tuna species such as yellowfin (*Thunnus albacares*), albacore (*Thunnus alalunga*) and kawakawa (*Euthynnus affinis*), SMRs are around 350 to 400 mg O_2/kg/h and are 2 to 10 times those of most active fish species such

as sockeye salmon (*Oncorhynchus nerka*), yellowtail (*Seriola quinqueradiata*), mackerel (*Scomber japonicas*) and trout (*Oncorhynchus mykiss*) (see Korsmeyer and Dewar 2001). Routine metabolic rates of SBT swimming at 1.1 body lengths per second are 460 ± 34.9 mg O_2/kg/h (Fitzgibbon et al 2008) compared to smaller skipjack tuna (*Katsuwonus pelamis*, 540 ± 63 mg O_2/kg/h) and bonito (*Sarda chiliensis*, 427 ± 63 mg O_2/kg/h, see Freund 1999, in Korsmeyer and Dewar 2001).

To assist adequate oxygen uptake to support their high rates of aerobic metabolism tuna have a large gill surface area and a thin gill epithelium. Gill lamellae on adjacent filaments are fused, providing additional support for the higher ventilation volumes from continuous and rapid swimming. Likewise tuna have large hearts to assist oxygen delivery to the tissues, generating high blood pressures and cardiac output. Brill and Bushnell (2001) report ventilation volumes in skipjack and yellowfin tunas approximately 5 to 10 times those of other fish, substantially higher effectiveness of oxygen transfer from water in tunas (>60%) compared to trout (33%) and cardiac outputs approximately three times those of other fish.

Fish have large blocks of white (fast-twitch) muscle fibres for powerful short-burst swimming and smaller groups of red (slow-twitch) fibres for sustained aerobic swimming contractions. In tuna, red muscle fibres are highly internalised and associated with a vascular counter current heat exchange system (retia mirabilia) that conserves metabolic heat making it available to warm the musculature, viscera and vital sensory organs such as the eye and brain above ambient water temperature. SBT maintain basal and visceral temperatures from 4 to 10^{o}C above ambient at water temperatures up to 20^{o}C; red muscle tissue at approximately 30^{o}C (irrespective of water temperature) and white muscle at approximately 6^{o}C above ambient (Ellis 2013).

1.2 SBT FISHING

Meany (2001) documented the catch and quota allocation history of the global SBT fishery. Particular emphasis was given to Australia where commercial utilisation commenced with a troll fishery, drawing fishing lines either baited or with lures through the water off the south east coast in the mid 1930s. By the early 1950s a pole and live bait fishery was operating in the Great Australian Bight (GAB), out of Port Lincoln, South Australia (Figure 1.1).

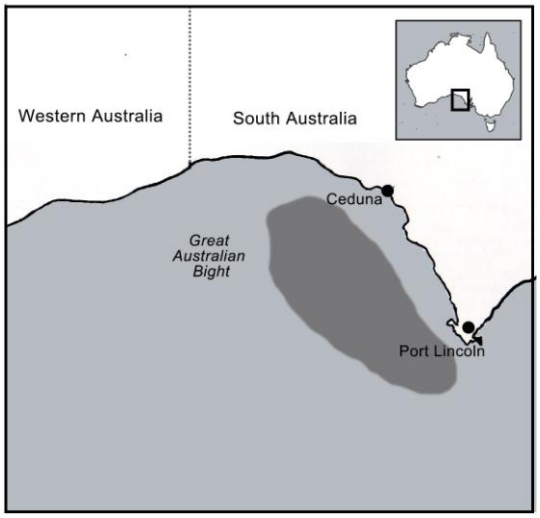


Figure 1.1 Location of principal surface fishery for SBT in GAB (shaded area)

From December to early April SBT migrate into the GAB supporting a surface fishery that initially targeted the high volume domestic and international canning market. The introduction of purse seine techniques in the mid 1970s resulted in greater catch efficiencies. Increasing effort and decreasing catches in the early 1980s resulted in a global total allowable catch (TAC) being introduced and set at 38,650 tonnes for 1985. The international management body, the Commission for the Conservation of Southern Bluefin Tuna (CCSBT) was established to safeguard the depleted stock. By 1989 the global TAC had been reduced further to 11,750 tonnes of which Australia's allocation was 5,265 tonnes. Figure 1.2 shows the Australian domestic catch history from 1952 to 2013.

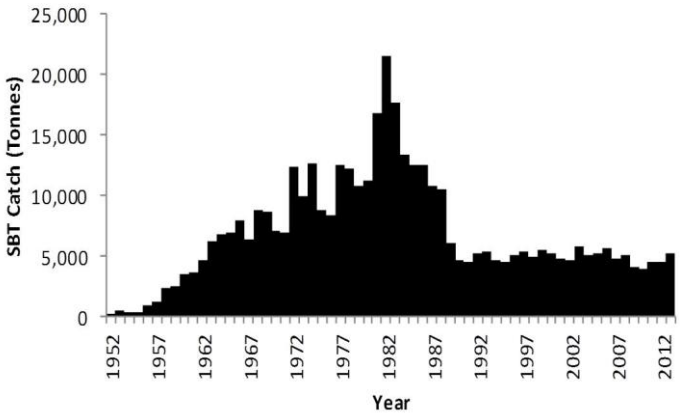


Figure 1.2 Domestic catch history (tonnes) for SBT from 1952 to 2013.
From Meany 2001 and Australian Fisheries Management Authority (pers comm)

This allocation remained until 2009 when it was reduced further to 4015 tonnes (ASBTIA[1] pers comm). With the quota enforced reductions in catch, greater economic benefit was sought by fishers in targeting the higher value Japanese sashimi markets. The preference of this market for higher oil/fat tuna opened the opportunity to hold over SBT and manipulate feed intake during a ranching period to maximise return. Since 2000 at least 95% of the domestic TAC each year has gone into the tuna ranching sector (ASBTIA pers comm). The improving status of the global stock has been acknowledged by CCSBT with modest increases to the domestic TAC since the 2009 cut (4528 tonnes in 2012, 4698 tonnes in 2013, 5147 tonnes in 2014 and 5665 tonnes for 2015-2017).

Unless otherwise acknowledged, the following details on ranching comes from the author's own experience in having managed Lincoln Marine Farm (LMF), the first commercial SBT ranching operation in 1992, and managing other ranching operations, South Australian Marine Farm (SAMF) and Marnikol Fisheries, up to and including the present time.

1.3 SBT RANCHING

Commercial SBT ranching began in 1992 following a successful collaborative research trial in 1991 between the then Tuna Boat Owners Association of Australia (now ASBTIA) and the Japanese Overseas Fishery Cooperation Foundation. SBT were poled onboard fishing vessels and transferred via canvas slings into 10,000 to 20,000L bait tanks through which seawater was pumped. Following a four to six hour trip back to Port Lincoln they were removed from the tanks individually using the vessel's boom with a canvas brail and released into grow-out cages. Sixty to 150 SBT were transported on each trip and less than 5% of the Australian quota was ranched in 1992. SBT were held and fed for up to six months before harvest. Mortality during the transport, transfer and grow-out period was up to 35% of fish stocked (LMF pers comm).

Husbandry practices at first developed through the 'bush' engineering skills of the original fishers and staff. In 1993 several operators began trials with various sized tow cages to bring back SBT from the fishing ground in larger numbers. SBT were still poled onboard the fishing vessel and then transferred via canvas slings to the tow cage adjacent. A swim through gate was developed to allow SBT to be transferred between the tow cage and respective grow-out cages. The number of SBT transported by this method increased to several thousands per trip.

[1] Australian Southern Bluefin Tuna Industry Association

The economic prospects of the industry hinged on ranching as much of the available quota as possible, and emphasis was placed on getting greater numbers of SBT back, and into ranching cages, more economically and with lower mortalities. By 1994 the practice of encircling SBT in a purse seine net and swimming them into the tow cage had evolved to reduce physical handling during capture. Though there have been continued refinements in net and tow cage design, deployment and operation since, this purse seine capture and tow technique has remained essentially the same up to the present day.

1.4 SBT RANCHING RESEARCH

Through trial and error, the initial years of SBT ranching returned continued improvements in mortality, production and operating costs (LMF, SAMF pers comm). Formal research and development (R&D) on nutrition (primarily on the development of a manufactured feed) was conducted through the initial Cooperative Research Centre for Aquaculture (1994-2000). The onset of unexplained mortalities in 1993 instigated formal epidemiological investigations for the first time. Mortalities up to 15% (SAMF pers comm) in that year were attributed to the scuticociliate *Uronema nigricans* (see Munday et al 1997). This episode, combined with a catastrophic event in April 1996 (Munday and Hallegraeff 1998, mortalities up to 65%: ASBTIA pers comm), highlighted to the industry the need to broaden its R&D efforts.

Health was an obvious and immediate focal area and was subsequently combined with nutrition, product quality, biomass production and environmental sustainability in the SBT portfolios of the Aquafin Cooperative Research Centre for Sustainable Aquaculture (Aquafin CRC) which operated from 2001 to 2008. Table 1.1 provides a summary of the principal research efforts undertaken for SBT in the fields of the environment, nutrition, husbandry, product quality/residues and physiology.

Environmental research has concentrated on benthic communities and sediment fluxes, and the oceanographic conditions and carrying capacity of the ranching region. Nutritional studies involved the dietary requirements of SBT, development and delivery of a suitable manufactured feed and feed supplements, baitfish profiles/use and bioenergetics models. Innovation in handling and cage management practices principally has been the realm of industry participants, however formal investigations in biofouling management and extended grow-out (long-term holding) have been undertaken.

Table 1.1 Focus of general (non-health) SBT ranching research and development.
DNA = Deoxyribonucleic acid, NIR = Near Infrared Spectroscopy, TBARS = Thiobarbituric acid reactive substances, K values = measure of muscle nucleotide breakdown, T:C ratios = Trypsin:Chymotrypsin ratios, YTK= yellowtail kingfish (*Seriola lalandi*), DHA = Docosahexaenoic acid, EPA = Eicosapentaenoic acid, PCB = polychlorinated biphenyl PCDD/F = polychlorinated dibenzo-p-dioxin and dibenzofuran, RMR = Routine metabolic rate, SMR = Standard metabolic rate

Focus	Source
ENVIRONMENT	
Benthic assessment, DNA probes, indicator taxa	Loo et al 2006
Nitrogen modelling, benthic fluxes, sedimentation	Fernandes et al 2007 a
Nitrogen/phosphorus leaching pellets, feed, faeces	Fernandes et al 2007b,
Sediments, nutrients, benthic metabolism, fallowing, fouling	Fernandes et al 2007c
Risks, oceanography, telemetry, seabirds, carrying capacity	Tanner (Ed) 2007
Hydrodynamic/biogeochemical models, sediments, plankton	Tanner and Volkman (Eds) 2009
NUTRITION	
Salmon surrogate, *in vitro/in vivo* protein digestibility	Bransden et al 2001
Pellets, juvenile growth, condition, water temperature	Glencross et al 2002
Bioenergetics, visceral temperature, archival tags	Gunn et al 2002
Nutritional requirements, manufactured feed, growth	Van Barneveld et al 2003
Pellets, weaning, markets, feed delivery, shelf stable	Gordon et al 2006
Baitfish profiles, vitamins, handling/oxidation, NIR testing	Buchanan 2007
Feed handling, vitamin/nucleotide loss, rancidity, TBARS	Musgrove et al 2007
High throughput injection, liquid vitamin premix supplements	D'Antignana et al 2008
Feed quality, vitamins, histamines, K values, TBARS	Musgrove et al 2011
Enzymes, T:C ratios, diet, caecum/stomach conditions	Leef et al 2012
HUSBANDRY	
Stereo-video measurement of length and body depth	Harvey et al 2003
Biofouling, water/O_2 exchange, regulation, treatment	Rough et al 2009, Ellis et al 2009a
18 mth growout, parasites, performance, quality, environment	Ellis et al 2009b
PRODUCT QUALITY	
Fortified pellet diets, antioxidants, flesh shelf life	Buchanan and Thomas 2008
Diet, flesh quality, vitamins, pellets, YTK surrogate, cell lines	Thomas et al 2009
Residues	
Mercury accumulation, growth dilution, lipid depletion	Balshaw et al 2008a, Balshaw et al 2008b
Mercury DHA/EPA concentration, cross carcass variation	Balshaw et al 2012
PCB, PCDD/F congeners in fillets, 5 mth and 18 mth growout	Phua et al 2008
Baitfish, pellets, cross carcass sampling, dioxin model	Padula et al 2004
Comparative chemical residues in wild and ranched fish	Padula et al 2008
PHYSIOLOGY	
Metabolism, mesocosm respirometer, respiratory tags	Musgrove and Fitzgibbon 2006
Metabolism, swimming speed, feed lipid content	Fitzgibbon and Seymour 2009
O_2 consumption, metabolic rate, feed ration	Fitzgibbon et al 2007
O_2 consumption, RMR, SMR, mesocosm respirometer	Fitzgibbon et al 2008
Respiration, blood equilibrium curves with temperature	Clark et al 2008
Visceral warming, archival tags, dietary energy	Ellis 2013

Improving flesh characteristics and shelf life and the sampling and management of chemical residues in the flesh have been the focus of product quality research. Physiological studies have measured the metabolic rates and investigated the thermoenergetics of SBT. Of greater interest for this study is the suite of health related investigations summarized in Table 1.2.

Table 1.2 Focus of health related SBT ranching research and development
DNA = Deoxyribonucleic acid, qPCR = quantitative Polymerase Chain Reaction, SYBR green = proprietary dye for staining nucleic acid, Ig = Immunoglobulin, ELISA = Enzyme-linked immunosorbent assay, Hb = Haemoglobin, LPS = Lipopolysaccharide

Focus	Source
General	
Qualitative health risk assessment	Nowak et al 2003
Blood cell types, reference ranges, cytochemistry, packed cell volume	Rough et al 2005
Cardicola epidemiology, sampling and laboratory diagnostics, surveillance program, long-term holding, biofouling	Nowak et al 2007
Immunohistochemistry, lipoma, neurofibrosarcoma	Johnston et al 2008
Offshore holding, mortality, condition, parasites, humoral indicators	Kirchhoff et al 2011a
Cell line development, virus surveillance	Crane & Williams 2005
Immortal cell line development	Bain et al 2013
Culturable microbiota, potential pathogens, post mortem changes	Valdenegro-Vega et al 2013
Wild fish blood variables, parasitic loads	Kirchhoff et al 2014
Parasites	
Scuticociliate *Uronema nigricans*, pathogenesis	Munday et al 1997
Digenea Aporocotylidae, *Cardicola forsteri*, taxonomy	Cribb et al 2000
Farmed / wild fish histology, heart inflammatory response	Colquitt et al 2001
Cardicola forsteri prevalence, abundance, condition index	Aiken et al 2006
Cosmopolitan platyhelminth parasites, host migration, molecular evidence	Aiken et al 2007
Copepods (*Pseudocycnus*, *Euryphorus*), flatworm *Hexostoma* ectoparasites	Hayward et al 2007
PCR detection *Uronema*, *Cardicola*, *Hexostoma* environment samples	Bott et al 2008
Copepod prevalence/abundance, eye damage, condition	Hayward et al 2008a
Seasonal copepod prevalence/abundance, eye damage	Hayward et al 2008b
Cardicola infection modelling, infection peaks post transfer	Aiken et al 2009
Sea lice, seasonal epizootics, long-term holding, condition, eye damage	Hayward et al 2009
Cardicola forsteri mortality, branchitis, myocarditis	Dennis et al 2010
Sea lice, blood fluke epizootics, mortality at 7 weeks	Hayward et al 2010
Sea lice reservoirs, chalimus larvae, Degen's leatherjackets	Hayward et al 2011
Cardicola, terebellid hosts, DNA sequencing, small cerceria	Cribb et al 2011
Cardicola eggs in gills, intensity in gill arches, sectioning depth	Dos Santos et al 2012
Blood flukes, antihelmintic, praziquantel, eggs in heart and gill	Hardy-Smith et al 2012
Cardicola intermediate host, *Uronema*, *Caligus* management, Immuno-stimulants, vitamins, antihelmintics	Nowak et al (Eds) 2013
Cardicola spp. qPCR detection, non invasive, SYBR green, TaqMan	Polinski et al 2013a
Cardicola forsteri epidemiology, company differences in infection	Aiken et al 2015
Immunology	
Wild fish, grow-out, Ig, lysozyme, complement	Watts 2000, Watts et al 2002
Blood flukes, antibody response, ELISA, interannual difference	Aiken et al 2008
Immunostimulants, condition, humoral indicators	Kirchhoff et al 2011b
Early grow-out, immune response, condition, Hb	Kirchhoff et al 2011c
Blood fluke prevalence/intensity, humoral indicators	Kirchhoff et al 2012
Cytokine transcription in leucocytes, head kidney, LPS/heat shock stimulation	Polinski et al 2013b
Stress	
Mortality, towing, parasites, microbiology, histology, haematology	Nowak et al 2009a,
Mortality spike, towing, haemotology, faeces/water cortisol,	Nowak et al 2010

The majority of work has focused on parasitology of SBT, the associated immune responses and impact on survival and the development of ameliorative measures. Particular focus has been on the life cycle of blood flukes (*Cardicola* spp.) and the epidemiology and treatment of its infection of SBT.

Tables 1.1 and 1.2 indicate that there has been limited R&D effort for the initial tow, transfer and early grow-out stage of the SBT ranching cycle. In particular, understanding of the stress responses of SBT associated with their coping with industry's current husbandry practices was limited.

1.5 FISH STRESS

General reviews of the stress response in fish are provided by Wendelaar Bonga (1997), Iwama et al (1997), Iwama (1998), Barton (2002) and Pankhurst (2011). Table 1.3, after Ellis et al (2012), summarises the principal responses and groups them into primary, secondary and tertiary categories. A synopsis of stress response mechanisms is provided in this section to support the choice of indicators investigated in this study. Selected indicators are highlighted in Table 1.3 and in the immediately ensuing discussion below. In this section general reference to results in other fish is provided and specific detail of other work on stress indicators in tuna is provided in Chapter 3.

The formal definition of stress adopted here is that of Iwama (1998), being 'the response of the cell, or organism, to any demand placed on it such that it causes an extension of a physiological state beyond the normal resting state'. Responses can range from simple behaviours to more complex physical and physiological processes involving the neuroendocrine and immune systems in order to free up energy resources to deal with the stressor and move back towards homeostasis. Table 1.4 provides a summary of selected research published since 2000 on fish stress indicators of interest here.

Avoidance behavior is an obvious primary response and may reduce the subsequent energy demands on the organism. Flik et al (2006) provide a detailed review of the primary physiological response which entails the release of signal molecules such as the catecholamines, epinephrine and norepinephrine, adrenocorticotropic hormone (ACTH) and melanocyte stimulating hormone (MSH). Multiple inputs from sensory systems continuously monitoring and evaluating the condition of the fish are received and integrated in hypothalamic nuclei. The release of corticotropin-releasing factor (CRF) from the hypothalamic nucleus preopticus stimulates the production of ACTH in the pituitary, which in turn results in the production of **cortisol** by the inter-renal tissue located in the head kidney (fish lack an adrenal gland). Catecholamine release is a very quick, neurological response making it very difficult to measure blood concentrations unaffected by sampling itself (Ellis et al 2012).

Table 1.3 Examples of the primary, secondary and tertiary stress response indicators in fish. Modified from Ellis et al (2012). ACTH = Adrenocorticotropic hormone, MSH = Melanocyte Stimulating Hormone, FCR = food conversion ratio. Parameters used in this study are in red

	STRESS RESPONSE	PRIMARY	SECONDARY	TERTIARY
NEUROENDOCRINE	Endocrine	Catecholamines ACTH, ***Cortisol*** MSH	Growth hormone	
	Molecular	Glucocorticoid receptors		
	Neurotransmitters	Serotonin		
PHYSIOLOGICAL	Biochemical		Acute phase proteins ***Plasma glucose***, ***lactate***, cholesterol Liver&muscle glycogen adenylate energy charge	
	Haematological		Haematocrit, leucocrit, erythrocyte/leucocyte numbers & ration, thrombocyte numbers, blood clotting time, ***haemoglobin***, ***pH***	
	Hydromineral		Plasma chloride, sodium, potassium, protein, ***osmolality***	
	Immunological		Cytokine release, Phagocytic index, oxidative burst, ***lysozyme***, ***IgM*** pinocytosis, ***complement***	
PHYSICAL	Histopathology	Interrenal cell size, number and nuclear diameter	Gastric tissue morphology, gill chloride cells, epidermal mucus cells	***Melanomacrophage centres***
	Biometric		Skin colour	Condition factor, organosomatic indices
	Behavioural	Escape response	Ventilation rate	Food intake, activity, space/refuge use, shoaling/schooling, aggression, reproductive behaviour
	Performance measures			Growth ***IGF***, FCR, size variation, disease incidence, ***mortality***, reproductive performance

Table 1.4 Stress responses in fish
[A] converted from nmol/L cortisol where nmol/L / 2.76 = ng/mL, [B] converted from mg/dL glucose where mg/dL x 0.055 = mmol/L, [*] approximate values transposed from graphical presentation in results of cited publications, T. = Temperature, (J) = Juveniles, Ant. = Antarctic

Indicator Stressor	Species	Pre stress	Post stress	Source
Cortisol				
Handling	Coral trout *Plectropomus leopardus*	4 ng/mL*	70.5 ng/mL	Firsch & Anderson 2000
Transport	Sturgeon (J) *Acipenser brevirostrum*	17.5 ng/mL*	14 ng/mL	Beyea et al 2005
Anaesthesia/ Transport	Atlantic salmon *Salmo salar*	71 ng/mL[A]	24 ng/mL[A]	Sandodden et al 2001
Confinement	Carp *Cyprinus carpio*	8 ng/mL*	185 ng/mL*	Ruane et al 2002
	Atlantic cod (J) G*adus morhua*	5 ng/mL*	95 ng/mL*	King & Berlinsky 2006
Cage net colour	Snapper (J) *Pagrus auratus*	3.9 ng/mL	10.2 ng/mL	Doolan et al 2008
Fin ablation	Tiger pufferfish (J) *Takifugu rubripes*	14 ng/mL	219 ng/mL	Hosoya et al 2008
High T.	Atlantic cod (J) *Gadus morhua*	< 10 ng/mL*	448 ng/mL	Pérez-Casanova et al 2008
Higher density	Atlantic salmon (parr) *Salmo salar*	17.5 ng/mL	86.6 ng/mL	Basrur et al 2010
	Sole (J) *Solea senegalensis*	7.5 ng/mL*	30 ng/mL*	Costas et al 2013
	Jade perch *Scortum barcoo*	130 ng/mL*	160 ng/mL*	Luo et al 2013
	Sturgeon (J) *Scaphirhynchus albus*	5 ng/mL*	20 ng/mL*	Nelson & Small 2014
Starvation	European eel (J) *Anguilla anguilla*	80.1 ng/mL	357.9 ng/mL	Caruso et al 2010
Ectoparasities	Artic charr *Salvelinus alpinus*	25 ng/mL*	145 ng/mL*	Tveiten et al 2010
Higher salinity	Grouper *Epinephelus malabaricus*	5.9 ng/mL[A]	35.3 ng/mL[A]	Tsui et al 2012
Air exposure	Wolffish (J) *Anarhichas minor*	5 ng/mL*	27 ng/mL*	Le François et al 2013
Copper exposure	Sturgeon (J) *Acipenser persicus*	15 ng/mL*	51 ng/mL*	Zahedi et al 2014
Glucose				
Handling	Coral trout *Plectropomus leopardus*	2 mmol/L*	7.56 mmol/L	Firsch & Anderson 2000
Higher density	Carp *Cyprinus carpio*	2.1 mmol/L*	3.6 mmol/L*	Ruane et al 2002
	Atlantic salmon (parr) *Salmo salar*	5.1 mmol/L	8.0 mmol/L	Basrur et al 2010
	Sole (J) *Solea senegalensis*	5.0 mmol/L	8.1 mmol/L	Costas et al 2013
Salinity change	Grouper *Epinephelus malabaricus*	1.26 mmol/L	2.77 mmol/L	Tsui et al 2012
Copper exposure	Sturgeon (J) *Acipenser persicus*	2.94 mmol/L[B]	4.49 mmol/L[B]	Zahedi et al 2014
Lactate Handling Transport	Coral trout *Plectropomus leopardus*	1 mmol/L*	4.14 mmol/L	Firsch & Anderson 2000
Higher density	Atlantic salmon (parr) *Salmo salar*	4.0 mmol/L	9.5 mmol/L	Basrur et al 2010
	Sole (J) *Solea senegalensis*	5.3 mmol/L	9.6 mmol/L	Costas et al 2013
Higher salinity	Grouper *Epinephelus malabaricus*	1.88 mmol/L	0.95 mmol/L*	Tsui et al 2012
Lysozyme				
Higher density	Sole (J) *Solea senegalensis*	17.5 ug/mL*	10.5 ug/mL*	Costas et al 2013
	Jade perch *Scortum barcoo*	14.5 ug/mL*	12 ug/mL*	Luo et al 2013

Table 1.4 cont				
Complement				
Higher density	Sole (J) *Solea senegalensis*	205 units/mL*	140 units/mL*	Costas et al 2013
Haemoglobin				
Handling/transport	Sturgeon (J) *Acipenser brevirostrum*	5.28 g/dL	7.0 g/dL*	Beyea et al 2005
Osmolality				
Handling	Sturgeon (J) *Acipenser brevirostrum*	277*	288*	Beyea et al 2005
Higher T.	Ant. fish *Pagothenia borchgrevinki*	574	629	Lowe and Davison 2005
Units mOsmol/kg/hr	Ant. fish *Trematomus bernacchii*	561	608	Lowe and Davison 2005

Needing to be first produced, cortisol blood levels typically take a few minutes to rise, reducing the influence of sampling (Molinero et al 1997) and enhancing its potential use as a stress indicator. Table 1.4 lists a number of stressors in a wide range of different fish that results in an elevated cortisol level. Cortisol influences a variety of physiological functions in fish, a primary outcome of which is the redirection of energy flows for optimal performance under conditions where homeostasis may be or become at risk.

Cortisol has a mediating role in various metabolic and ion uptake processes, influencing blood **glucose** levels, growth potential as mediated by **IGF-1** levels (McCormick et al 1998), **osmolality** (McCormick 2001) and multiple aspects of the innate immune system (Pankhurst 2011). Handling, increased stocking density, changes in salinity and exposure to a pollutant (copper) have been shown to increase glucose levels (Table 1.4). Similarly, handling and higher temperatures have been reported as increasing blood osmolality.

A major source of glucose comes from either the glycogen stored in the liver (Polakof et al 2012) which is liberated through glycogenolysis (direct cleavage of glycogen), or gluconeogenesis (direct synthesis of glucose from non-carbohydrate carbon structures such as glycerol, pyruvate and lactate**)**. Levels of pyruvate and its metabolite **lactate** may also be expected to alter as elevated glucose levels are utilised during glycolysis to provide the energy source for vital organs such as the brain, gills and muscle to deal with the stressor. Not surprisingly then, Table 1.4 describes an increase in blood lactate levels from a similar range of stressors producing a glucose response.

The enzyme **lysozyme** is a component of the innate immune system that forms a biochemical barrier to antigenic challenge by hydrolysing bonds of bacterial cell wall peptidoglycans (Magnadóttir 2006). The suite of proteins making up the **complement** system has an important

role in non-self recognition providing defence against a wide range of antigens (Boshra et al 2006). Natural antibodies such as **IgM** provide an early and broad protection against antigens via activation of the complement system and the enhancement of the adaptive antibody response (Whyte 2007). A key attribute of non-self recognition is the pattern recognition receptors of these components of the innate immune system, the binding ability of which can be influenced by the hormones released by the primary neuroendocrine stress response. Catchecholamines bind with adrenoceptors present in the cells of the immune system and cortisol modifies gene expression and thus influences the secretion of pro and anti-inflammatory cytokines, as well as immunoglobulin (Magnadóttir 2006, Pankhurst 2011). As a result, chronic stress is well documented as having an inhibitory influence on the immune response of fish (Pankhurst 2011, Nardocci et al 2014). Stress hormones influence oxygen uptake (Ellis et al 2012) of which **haemoglobin** concentrations can be a useful measure. Table 1.4 provides examples of each of these indicators responding to various stressors of fish.

Melanomacrophage centres (MMCs) in the haemopoietic tissue of some major organs also have a role in immune system defence, being deposition sites for intracellular bacteria and involved in antigen trapping and presentation to immune cells, and in the storage of degenerate material or material for recycling such as iron (Wolke 1992, Agius and Roberts 2003). MMCs accumulate pigments associated with tissue and cellular deterioration (Agius and Agbede 1984); lipofuscin from damage to cell membranes; haemosiderin from the ageing/damage to red blood cells and melanin from damaged melanin-containing cells.

Some authors have debated the role of MMCs as biomarkers of potential environmental stressors. Wolke (1992) questioned their value as 'monitors' due to the scarcity of investigations of controlled exposure studies. Haaparanta et al (1996) found no agreement with the pattern observed in the number or areal cover of MMCs of fish from four lakes providing a gradation in water quality and Bols et al (2001) concluded that the meaning of changes to MMCs in field studies of toxicant induced changes is not clear. However the potential of MMCs as indicators of the overall health of fish appears to have gained credence in most recent years, particularly with respect to feed status (Mizuno et al 2002, Agius and Roberts 2003, Hur et al 2006, Rios et al 2007, Reyes-Becerril et al 2014), anthropogenic pollutants (Suresh 2009, Ali et al 2014, Passantino et al 2013, Taheri et al in press) and environmental toxins (Pronina et al 2014).

1.6 STUDY SCOPE

This thesis investigates the above highlighted aspects of the stress and immune responses of southern bluefin tuna (*Thunnus maccoyii* Castleneau) held under ranching conditions. The aim was to pursue a base of knowledge on health impacts of the components of ranching to provide a foundation for the eventual improvement in production capacity through appropriate ameliorative measures where possible.

It provided the first opportunity to look at various aspects of the period from towing through early grow-out of five companies over a six year period (2003 to 2008). For confidentiality purposes these companies are referred to as Companies 1 to 5 hereafter. Unfortunately the sale of one company through the study period precluded the continuity in data source and another company was enlisted to fill the void.

In order to provide a context for subsequent discussions of the potential influence of husbandry on mortality and stress, a review of capture-based operations, including those for bluefin tuna, elsewhere in the world is provided at the start of Chapter 2. That is followed by an analysis of the SBT industry's towing practices and their influence on the mortalities in the first three months of grow-out. The objective was to highlight tow parameters that would provide optimal results in terms of stock survival in these early months.

Investigations of the stress and immune response indicators of ranched SBT are detailed in Chapter 3 and the impact on stress of holding ranched SBT over a substantially greater time period (18 months compared to the industry practice of 6 to 8 months) is considered in Chapter 4. Those indicators of greatest utility in influencing/predicting SBT health were of interest within both the standard ranching time frame and longer-term captive holding.

The final research Chapter (5) investigates how the melanomacrophage centre profiles in some major organs vary over the ranching cycle. Of interest was whether MMC profiles might be useful in providing a measure of the health impacts of ranching integrated over an entire production period. Chapter 6 provides a general discussion and the associated implications of the study's results for SBT ranching.

CHAPTER 2 SBT CAPTURE AND TOWING

2.1 INTRODUCTION

SBT ranching is one of a number of forms of capture-based aquaculture where seed-stock for grow-out is sourced from wild populations. This sector has had a long history worldwide and involved various species/groups. Table 2.1 provides a list of substantive capture-based operations although in some cases the present day split between traditional capture-based seed-stock supply in these fisheries and now hatchery supply is unclear.

Table 2.1 Examples of capture-based aquaculture operations

Common name	*Species*/Taxa	Major Regions/Countries	Source
Carp	Cyprinidae	Asia	Rahman 2008
Catfish	*Clarias* spp.	Cameroon	Pouomogne 2008
Eels	*Anguilla* spp.	Asia, Europe, North America, Japan	Ottolenghi et al 2004
Groupers	*Epinephelus* spp.	Asia, South-east Asia, USA, Carribean, Australia	Ottolenghi et al 2004
Milkfish	*Chanos chanos*	South-east Asia, Pacific Islands	Martinez et al 2006
Mullets	Mugilidae	Egypt	Saleh 2008
Shrimp	Penaeidae	South America, Asia	Ottolenghi et al 2004
Snakeheads	*Channa* spp.	South-east Asia	Poulsen et al 2008
Tunas	*Thunnus* spp.	Australia, Japan, Europe, Mexico, Morocco	Ottolenghi et al 2004
Yellowtails	*Seriola* spp.	Japan, Asia	Nakada 2008

The majority of capture-based aquaculture is in developing countries and essentially is artisanal, even though significant production volumes often are involved. The freshwater species are mainly monsoonal spawners and fry are collected in association with seasonal flooding. Carp fry are collected by fixed nets or portable scoop or seine nets (Rahman 2008). Snakehead fry also are collected by various nets but also retained in floodplain based trap ponds or rice fields following flooding (Poulsen et al 2008). Catfish seed-stock in Cameroon is sourced by collection ponds (Pouomogne 2008). These fish are transported to grow-out systems of ponds or small net cages in small containers or vehicle based tanks, and transport is generally of both a short distance and duration. There are few comparisons that can be drawn meaningfully between these freshwater, artisanal aquaculture operations and SBT ranching. The marine based mullet operations of Egypt are also of limited purpose in this context due to its artisanal nature. The other marine species listed in Table 2.1 however are of relevance and particularly the capture-based aquaculture of other tunas (Section 2.1.1).

In Australia the yellowtail kingfish (*Seriola lalandi*) is fished by the Ocean Trap and Line Fishery of NSW (NSW DPI 2009) and cultured in hatchery supplied sea cage operations in Spencer Gulf, South Australia (FRDC 2013). No capture-based aquaculture operations exist. However various yellowtail species (*Seriola* spp.) are cultured extensively elsewhere and particularly in Japan from wild caught seed-stock (Ottolenghi et al 2004, Nakada 2008). Wild juveniles (<50 g) are harvested with nets, often in association with floating seaweeds in which they take refuge and feed upon plankton and other small fish. Transportation to grow-out net cages is undertaken in the holding tanks of fishing boats, similar to the vessel bait tank transport initially used for SBT (Section 1.3). Mortality during the period from capture to the start of farming can be 20 to 30%, and with suitable handling, less than 2% from then on to harvest (Nakada 2008).

Capture-based grouper culture operations have an 8 month culture period comparable to that of ranched SBT and have high total mortalities of 50% or more (Ottolenghi et al 2004). However in this sector handling and transport can be sub-optimal through the use of push nets and the need to maintain fry in plastic bags (with or without supplemented oxygen) for up to 12 hours during sometimes international shipping. Under such constraints the high mortalities can be understood.

2.1.1 Capture-based aquaculture of bluefin tunas

Canada

Bluefin tuna were held first commercially under captive conditions in Canada in the 1960s (Miyake et al 2003). Local fishermen with Japanese input placed Atlantic bluefin tuna (ABT; *Thunnus thynnus*) into net pens, holding and feeding them baitfish for several months before sale. ABT were captured using set trap nets (traditionally used for fishing for tuna) which involve a series of leading net ‘fences’ directing fish into an inner chamber, often with a meshed bottom that can be shallowed up to restrict and access the fish (United Nations 1958). A maximum production of 948 tonnes was achieved in 1977 but that fell drastically to 72 individuals in 1979 and virtually zero in 1984 (Ottolenghi et al 2004). Despite a slight improvement in production for a couple of years in the mid 1990s (under 80 tonnes) an apparent change in the migratory path of the fished stock resulted in the operations closing.

Japan

Pacific bluefin tuna (PBT; *Thunnus orientalis*) aquaculture operations commenced in Japan in the 1970s based on growing out 0-year old juveniles (Ottolenghi et al 2004, Masuma et al 2008). Juveniles were caught with barbless trolling lines and held in pens of net and rope held up by floats. As well as fattening fish for sale, a corollary objective has been to breed PBT to provide hatchery sourced stock for grow-out.

Spontaneous spawning of PBT in captivity was first achieved at Kinki University in 1979 (Kaji 2003) although progress in closing the life cycle has been slow with an artificially reared PBT not being induced to spawn until 2002 (Ottolenghi et al 2004). Numerous federal and prefectural governments, universities and private companies in Japan together have collected from zero to 500 million PBT eggs annually from broodstock operations (Masuma et al 2008, Masuma et al 2011). Tens of thousands of fingerlings of 35 to 50 mm have been produced per season although hatchery and nursery survival have been low (Masuma et al 2011, Ishibashi et al 2013) and juvenile PBT placed into sea net cages have under 50% survival in the first 30 days (Okada et al 2014).

Notwithstanding the 40 years of research on hatchery production, the Japanese PBT aquaculture sector has depended heavily on wild captured juveniles, with an estimated 150,000 to 200,000 0-year old juvenile PBT taken from the wild annually in the 2000s (Masuma et al 2008). Table 2.2 starkly illustrates the continuing economic dependence of the Japanese PBT aquaculture sector on the sourcing of wild caught juveniles (Japanese Fisheries Agency unpublished data). Over 97% of recent production routinely comes from wild sourced juveniles.

Set trap nets and trolling are traditional Japanese fishing methods yet account for only 12% and 13% of the Japanese PBT commercial catch. Purse seine boats account for the majority of the catch (55%), with the rest being taken by longline (9%), pole and line (6%) and others (5%; Suzuki and Kai 2012). However the large number of boats targeting juveniles for the PBT aquaculture sector (Table 2.2) suggests a preponderance of artisanal scale collectors trolling for juveniles and transporting small numbers to culture sites. As such, the Japanese capture-based sourcing of PBT for culture is of limited use for comparison with the capture and transport component of SBT of interest here.

Table 2.2 Japanese PBT farming data 2011 to 2014 with respect to the relative dependence on wild and hatchery sourced juveniles. Source Japanese Fisheries Agency unpublished data supplied by ASBTIA *figures in brackets indicate those restricted to only stocking hatchery sourced stock

	Year			
	2011	2012	2013	2014
Number of farming areas*	137	140	147 (30)	150 (36)
Number of cages*	1,031	1,191	1,362 (104)	1,375 (124)
Number of catching boats targeting juveniles	2,300	1,900	1,500	1,500
Total fish entering cages	753,000	473,000	611,000	519,000
Juveniles sourced from wild	539,000	205,000	347,000	221,000
Tonnes produced from wild sourced stock	NA	9,348	10,120	14,326
Juveniles sourced from hatcheries	214,000	268,000	264,000	298,000
Tonnes produced from hatchery sourced stock	NA	244	276	387
Total production from wild sourced stock (%)	NA	97.5	97.3	97.4

Mediterranean

Set trap nets were used in the Andalusian region of Spain in the 1980s to capture ABT and hold them for fattening (Ottolenghi 2008). Production however was modest with only two operations in Barbate and Ceuta transferring post-spawning ABT caught in traps to cages (FAO 2005). Significant volumes of cultured ABT were not produced in the Mediterranean until expatriate Croatian fishermen that had been part of the pioneering of SBT ranching in South Australia established ABT operations in 1996 in their country of birth (Miyake et al 2003, Katavic 2003a). Table 2.3 provides a summary of the increases in ABT aquaculture production of some Mediterranean countries from 1995 to 2004 (Cardia and Lovatelli 2007).

Table 2.3 ABT farming production 1995 – 2004 for some Mediterranean countries (Cardia and Lovatelli 2007). n.a. = not available, * = estimate only

Country	1995	1996	1997	1998	1999	2000	2001	2002	2003	2004
Croatia	-	-	390	400	672	1,200	2,500	3,971	4,679	3,777
Cyprus	-	-	-	-	-	-	-	-	-	1,370
Italy	-	-	-	-	-	-	800	1,800	1,700	n.a.
Malta	-	-	-	-	-	330	1,108	1,855	3,550	n.a.
Spain	n.a.	77	173	1,879	3,347	3,682	4,447	4,751	3,687	6,423
Tunisia	-	-	-	-	-	-	-	-	678	1,485
Turkey	-	-	-	-	-	260	3,800	3,300	4,100*	n.a.

The European Union and eight other Mediterranean countries have ABT quota (ICCAT 2015). By 2007 Libya, Greece, Morocco and Portugal had joined those in Table 2.3 in establishing ABT aquaculture operations (Ottolenghi 2008), and all but Portugal declared ABT landings in 2013, the bulk of which are caught for ABT aquaculture (ICCAT 2014).

The culture system developed in Australia for SBT and transferred to Croatia in 1996 was thence adopted by the majority of ABT operations in the Mediterranean in the late 1990s and early 2000s. Throughout the region traditional fishing techniques such as trolling and set net traps have been superseded by purse seine methods which recorded 88% of the Mediterranean catch in 2013, followed by longlines (6.6%) and set net traps (2.4%), with 3% sport fishing and miscellaneous others (Miyake et al 2010, ICCAT 2014).

As purse seine use and interest in holding over captured ABT increased in the 2000s the International Commission for the Conservation of Atlantic Tunas (ICCAT) acted in 2010 to enhance sustainability of the eastern Atlantic and Mediterranean fishery. The TAC was decreased from 32,000 tonnes to 13,500 tonnes, the use of purse seine capture was restricted to one month (May 15 to June 15) and the use of aerial spotter planes to locate fish was banned (Mylonas et al 2010). Other management measures restricted the size of ABT that could be taken to over 30 kg or with a fork length greater than 115 cm (ICCAT 2015). Concessions to the size restriction recognized more traditional fishing methods by having a minimum size for ABT of 8 kg or 75 cm fork length for fish caught by coastal artisanal fishers for fresh fish by baitboats, longliners and handliners. The same size concession was made for ABT caught in the Adriatic Sea for farming purposes in recognition of the Croatian aquaculture model. An incidental catch of a maximum 5% of the total catch of each purse seine fishing operation can be within the 8 to 30 kg and the 75 to 115 cm size limits (ICCAT 2015).

The smaller size of the ABT caught in the Adriatic for Croatian operations requires the fish to be held longer, often up to two years. Elsewhere in the Mediterranean the bigger ABT are held between 3 and 7 months only and ICCAT distinguishes between the farming of ABT in Croatia and the fattening of ABT elsewhere (Mylonas et al 2010). In regards the capture, towing and early grow-out of SBT of interest in this chapter, the Croatian operations involving similarly sized tuna are of more direct comparative use.

As indicated above, initial operations in Croatia were based on methods developed for SBT. One to two year old ABT were captured within the central Adriatic during the period June to October with purse seine boats and transferred into hexagonal Bridgestone tow cages for transport to grow-out sites among the islands off-shore Zadar to Split (Katavic et al 2003a). Initially, the same tow cages were used in Port Lincoln SBT operations however the high maintenance and component failure at sea of locally manufactured Bridgestone 'clones' (LMSC pers comm) resulted in more efficient, high-density polyethylene tow cages being developed.

Eighty percent of the Adriatic sourced ABT were less than 10 kg, 10 to 15% were 11 – 20 kg with 5 to 10% of bigger fish (Katavic 2003b). Approximately 80 to 100 tonnes of ABT were placed in tow cages and towed to the grow-out sites within 100 nautical miles (nm) of the fishing grounds at about 1.2 knots. Mortalities in the initial 'adaptation' month after capture were approximately 2%, and despite low mortalities of 0.6% in the next three months of an early trial (Katavic 2003a), were reported to be 3 to 5% over subsequent farming months during the late 1990s to mid 2000s (Katavic 2003b).

Like the PBT in Japan, considerable work has been undertaken on closing the life cycle of ABT in Europe to safeguard a seed-stock supply for grow-out. This has included major collaborative multi-disciplinary studies REPRO-DOTT and its successor SELFDOTT (Bridges et al 2003, Anon 2007, de la Gandara et al 2009). Captive held broodstock have been induced to spawn using a slow release implant containing a gonadotrophin releasing hormone agonist (Bridges et al 2009) and tens of millions of fertilized eggs have been collected for subsequent larval rearing (Caggiano et al 2009). Low survival of tuna larvae and fry in the first two months post hatch has limited the commercial application of the research undertaken to date (Mylonas et al 2010, Partridge 2013).

Mexico

From 1982 to 1995 the eastern Pacific Ocean fishery for PBT was dominated by the United States of America (US) catching 22,551 tonnes of PBT at an average 1,610 tonnes per annum (IATTC 2012a; range 508 to 4,920 tonnes). By comparison Mexico caught 2,510 tonnes over the same period at an average 179 tonnes per annum (range 0 to 676 tonnes). In 1996 PBT aquaculture operations commenced in Mexico, mimicking those in the Mediterranean with significant Australian management input during the early years (Sylvia et al 2003, ASBTIA pers comm). From then up to and including 2010, the US caught 14,800 tonnes at an average

987 tonnes per annum (range 60 to 4749 tonnes in 1996) whereas Mexico caught 57,947 tonnes at an average 3,863 tonnes per annum (IATTC 2012a). Like elsewhere where fishing methods focused on purse seine techniques to provide stock for grow-out, only 74 tonnes of the 48,387 tonnes of PBT caught by Mexico from 2001 to 2010 were by methods other than purse seining (IATTC 2012a).

A combination of water depth, fish behaviour and weather conditions contributed to inconsistent and unpredictable seasons in the early years of Mexican operations (Sylvia et al 2003), although the basic methodology reported is the same as that outlined above and described in greater detail in Section 2.1.2. PBT ranging in size from 15 to 45 kg are captured by purse seine boats, transferred into tow cages and towed distances of 50 to over 400 nm to grow-out sites for a 3 to 8 month production cycle in cages 40 to 50 m in diameter and 15 to 20 m in depth (Sylvia et al 2003, Zertuche-González et al 2008).

Syliva et al (2003) relate that weather events such as El Niño and hurricanes (Nora) led to high mortalities in the first years of Mexican operations. Initially mortalities during tows of 10 to 20% were experienced and 10 to 15% during grow-out (Zertuche-González et al 2008). By the mid 2000s these mortalities had reduced to 1 to 3% and 6% respectively. Sylvia et al (2003) reported that predators, particularly sea lions, contributed to stress (unspecified) and poor growth performance being common in most farms. At least a third and sometimes up to one half of PBT harvested had fresh or healing wounds from sea lion interactions. Recent concern for the sustainability of the PBT fisheries has led the Inter-American Tropical Tuna Commission (IATTC) to reduce the 2012 to 2013 PBT commercial catch limit in the eastern Pacific Ocean from 10,000 tonnes (IATTC 2012b) to 6,600 tonnes (IATTC 2014).

2.1.2 Capture-based aquaculture of SBT

Due to local convention SBT capture-based aquaculture operations have been referred to here as ranching rather than farming or fattening. A brief synopsis of SBT fishing and ranching in Australia was provided in Sections 1.2 and 1.3 respectively. From the discussion above it is obvious the SBT ‘model’ has formed the basis of the modern aquaculture operations of ABT in the Mediterranean and PBT in Mexico. An expanded description therefore is warranted.

The GAB fishing grounds extend up to 200 nautical miles (370 kms) from the ranching sites off shore Port Lincoln (refer Figure 1.1). Tow boats with one or two 40 to 50 m cages in tow take up to 5 days to travel to the fishing grounds. Spotter planes are used to direct the fishing

fleet to the appropriate areas. On the fishing ground purse seine net boat crew locate schools visually or with sonar and SBT are attracted and congregated using chum-feeding of live or frozen bait fish. Schools of up to 5000 SBT are encircled by the purse seine net and made ready for transfer. Once the net is closed the surface floats are held open by a number of small motorized tenders whilst awaiting the positioning of the tow cage. This ensures as much room as possible is maintained for the SBT.

Once the tow boat has brought the tow cage alongside, sea gates on both the tow cage net and the purse seine net are clipped together to provide an opening for transfer of up to 20 m in length and 6 to 8 m in depth. The SBT in the purse seine net are encouraged to swim through the sea gate into the tow cage by slowly reducing the volume of the net. This capture/transfer process is repeated several times until the required number of SBT is achieved in the tow cage and the tow back to ranching sites commences. Tow cages have an elaborate set up of counter weights (ranging from 100 to 500 kg each) and an accompanying system of surface and subsurface tow bridles (up to 14) to maintain net shape under tow.

With SBT in tow the return trip can take from 2 to 6 weeks depending on the distance of the capture sites and weather/sea conditions. Some companies feed the SBT on tow, others do not. The average weight of the SBT in the tow cage is calculated on arrival off shore Port Lincoln by measuring a hook-caught sample of 100 fish above a mandated 10 kg minimum weight. At the grow-out sites the at-sea process is reversed and SBT are forced to swim into the grow-out cages via the transfer gate by decreasing the depth of the tow cage net using dry-up ropes. Usually SBT are transferred in to 3 or more grow-out cages from each tow cage. All SBT entering grow-out cages are recorded on video as they swim through the transfer gate for subsequent counting. The compliance monitoring is supervised by AFMA representatives to record the quota taken. After transfer, SBT are fed once or twice per day, five or six days per week until harvest up to 8 months later.

Research on the closure of the SBT life cycle also has been undertaken (Stehr 2010). Many millions of fertilized eggs were produced in the late 2000s and millions of larvae hatched, some surviving up to 200 days post hatch. However like elsewhere, difficulties in larval rearing have prevented the commercialization of research up to date.

The summary of capture-based aquaculture operations above highlights the importance of suitable entrapment and transport methodologies for wild sourced seed-stock. Initial mortalities can be high if traditional fishing techniques are merely adopted for use (over 30% for yellowtail and SBT for line/hook capture and in-board transport in boat tanks). Techniques removing direct handling intuitively appear more suitable and purse seine entrapment and swim through transfer in tow cages reduced capture mortalities to approximately 2 to 3% for ABT and PBT.

Success in getting production to market, the fish farmer's challenge, reasonably can be expected to be a function of the efficacy of these initial capture/transport stages. Therefore the first step in describing the stress-related impacts in SBT ranching was to quantify the influence of these stages where possible. Mortality is a very obvious, albeit crude, measure of how well technology meets the biological requirements of SBT. A qualitative investigation of how various transport or tow variables influenced mortality was undertaken. Access to a number of operation's tow experience over a number of years was required to assess identified tow parameters.

2.2 METHODS

The study focused on the period 2003 to 2008 when increasing mortalities were experienced in ranching operations. Summary tow statistics for the industry over the period were obtained (ASBTIA pers comm). Tow and mortality data over the first three months of grow-out each year were recorded from participating companies. No size or growth data was available for inclusion in the study due to corporate sensitivities. Of particular interest in regards potential stressors throughout the capture and transport phases were the period of time through which newly caught SBT were held under presumed 'crowded' conditions, the extent of crowding and the speed of travel forced upon the captive SBT within the tow cage.

An additional identified potential stressor was the point of transfer at end tow into the grow-out cage. This is achieved by progressively raising the net bottom of the tow cage to force the SBT towards the transfer gate and through into the grow-out cage. The extent and rate of this net raising dictates the holding density of the SBT remaining in the tow cage and the comparative 'pressure' placed upon the SBT to swim through the transfer gate. The need to film and clearly identify and count each SBT on video for quota management mandates a consistent passage of SBT through the transfer gate however there is some discretion in what

constitutes a suitable rate of transfer. As a result there can be a wide range of lengths of time during transfers that SBT are subjected to elevated holding densities and forced activity. The influence of this transfer phase on subsequent mortalities was investigated.

Table 2.4 provides a list of the tow parameters recorded. The variables in red are derived from the primary data supplied by participating companies. Three companies contributed data for the entire study period. One contributed for all but the last year. A fifth company filled the void in 2008 and whilst being of interest in adding another operator for comparative purposes in that year, the loss of the fourth company restricted the extent of interpretations possible.

Table 2.4 Variables used in historic tow data analysis. Derived variables calculated from primary data are in red

Variable	Variable descriptor
Protec Tow Number	Number of tow cage as recorded by Protec
Cage Number (growout)	Number of holding cage fish transferred into
Diameter	Diameter of tow cage
Leadline depth	Depth of tow cage to leadline
Latitude	Latitude of capture area
Longitude	Longitude of capture area
1st fish capture	Date of 1st fish into tow cage
Last fish capture	Date of last fish into tow cage
Average weight	Average of 40 fish sample* taken with Protec
Average weight date	Date of 40 fish sample -- taken as end of tow
Tow length (days)	***No. days between 1st fish capture and Average weight date***
Tow distance	***Distance (nm) between capture lat/long and end of tow point***
Tow speed	***Tow distance / tow length / 24 hrs***
Tow density	***Density of fish in tow cage in kg/m^3***
Tow biomass	***Biomass of fish in tow cage in kg/m^3***
Number of fish in tow cage	Sum of fish transferred to holding cages plus tow mortalities
Number of tow mortalities	Number of mortalities in tow cage
Number of fish	Number of fish transferred to holding cage
Transfer date	Date of transfer into holding cage
Diameter of Holding cage	Diameter of holding cage
Leadline depth of Holding cage	Depth of holding cage to leadline
Start transfer	Time of starting transfer
Finish transfer	Time of finishing transfer
Transfer time	***Minutes between start and finish of transfer***
Mortalities weekly for 3 mths	Mortality in respective weeks to week 12

* 40 fish weight sample was used up to 2012. 100 fish sample thereafter

Data were received for each individual grow-out cage of the companies that had fish transferred into them over the review period. Where comparisons were made at a tow cage level, individual grow-out cage numbers and mortalities were summed and expressed as a total for the relevant tow cage. A geographic point approximately 1.5 nautical miles north east of Davidson Rock in the centre of the ranching zone was used as the end tow position for all tows. Note the term end tow is used hereafter to denote where the tow cages have reached the ranching zone immediately prior to transfers. The end tow sampling described in Chapter 3

was undertaken at the time of the average weight hook sampling for each tow (Section 2.1.2) and was within a few nautical miles of the geographic point described above.

Relationships between tows and variables were investigated using graphical, classification (hierarchical cluster analysis), regression techniques and correlation (Spearman's rho coefficient, r_s). A Spearman's r_s coefficient of 0 to 0.19 was considered a very weak relationship, 0.2 to 0.39 a weak relationship, 0.4 to 0.59 a moderate relationship, 0.60 to 0.79 a strong relationship and 0.8 to 1.0 a very strong relationship[2]. All statistical inference testing was undertaken using SPSS software.

2.3 RESULTS

2.3.1 Total industry mortalities

Mortality within capture-based aquaculture operations can occur during capture, transport and grow-out. With regards SBT ranching this applies respectively to the encircling by net/transfer of the schools into tow cages, the duration of the tow and from transfer into grow-out cage to harvest. Summary parameters for the capture and tow stage of the industry for 2001 to 2014 are provided in Table 2.5 (ASBTIA pers comm). Note actual fishing periods include the December of the year before.

Table 2.5 Historic capture and tow effort of SBT ranching industry (Source ASBTIA pers comm)
[A] Reduced due to payback for over catch in 2003; [B] Reduced quota due to cut in Total Allowable Catch; [C] Quota increased 2012 and 2014 fishing seasons

Year	Catch boats	Quota (T)	Catch mortality (no. of fish)	No. of Tows	Mortality (%) (pre-transfer)
2001	8	5161	71	38	0.42
2002	7	5234	61	40	0.34
2003	7	5375	82	38	0.37
2004	6	4860[A]	86	36	0.24
2005	7	5215	113	36	0.33
2006	6	5302	128	38	0.25
2007	6	5228	106	33	0.17
2008	6	5212	73	30	0.18
2009	7	5014	91	31	0.18
2010	5	3930[B]	68	23	0.21
2011	4	3872	71	24	0.23
2012	5	4465[C]	na	28	0.13
2013	5	4198	na	27	0.08
2014	5	5025	59	25	0.08

The catch efficacy of the SBT purse seine fleet is high with mortalities during catch ranging from an aggregated season total for all vessels of 59 to 128 individuals out of a range of approximately 212,000 to 354,000 SBT during the 2001 to 2014 capture seasons (ASBTIA

[2] Source: http://www.statstutor.ac.uk/resources/uploaded/spearmans.pdf

pers comm). Figure 2.1 shows all mortalities before transfer into grow-out cages for the total SBT ranching sector and indicates a continued improvement in the capture/transport phase of the sector over the last 15 years.

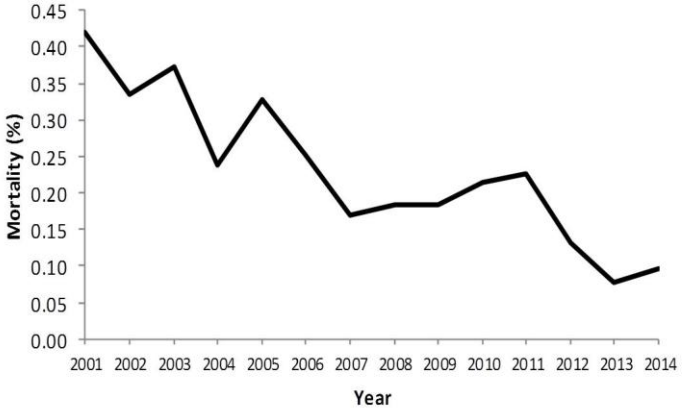


Figure 2.1 Pre transfer mortalities as percentage of SBT caught for 2001 to 2014 for the whole ranching sector (Source ASBTIA pers comm)

Figure 2.2 indicates that by 2001 grow-out mortalities for the whole industry had reduced to below 5% and remained below 4% until 2006. There was a marked increase in mortality in 2007 and 2008, the last two years of this study. Because of the spike in mortalities from 2006 to 2007, subsequent investigations of the capture/transport phases of SBT ranching delineated the 2003 to 2006 and the 2007 to 2008 periods.

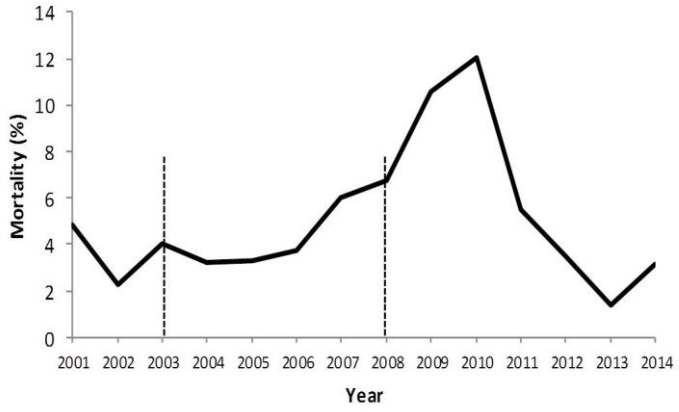


Figure 2.2 Grow-out mortalities as percentage of SBT transferred for 2001 to 2014 for the whole ranching sector (Source ASBTIA pers comm). Dashed lines delineate the period of years included in this study (2003 to 2008)

The dramatic increase in grow-out mortalities from 2006 to 2010 (3.6 to 13.5%) and the equally dramatic drop from then to 2013 and 2014 (1.3 and 3.2%) will be addressed in Section 2.4.

2.3.2 Early grow-out

The early grow-out period was of interest to try and elucidate the increasing trend in mortalities over the study period. Figure 2.3 shows the mortalities as a percentage of total SBT transferred for the contributing companies combined for the first 12 weeks post transfer for each of the years 2003 to 2008. There is a consistently higher mortality in the first week or two after transfer, followed by a gradual reduction over the next month. In all years mortalities again rose at 5 to 6 weeks, remaining elevated until 8 to 10 weeks post transfer. The spikes in mortalities at this stage of grow-out were greatest in 2007 and 2008.

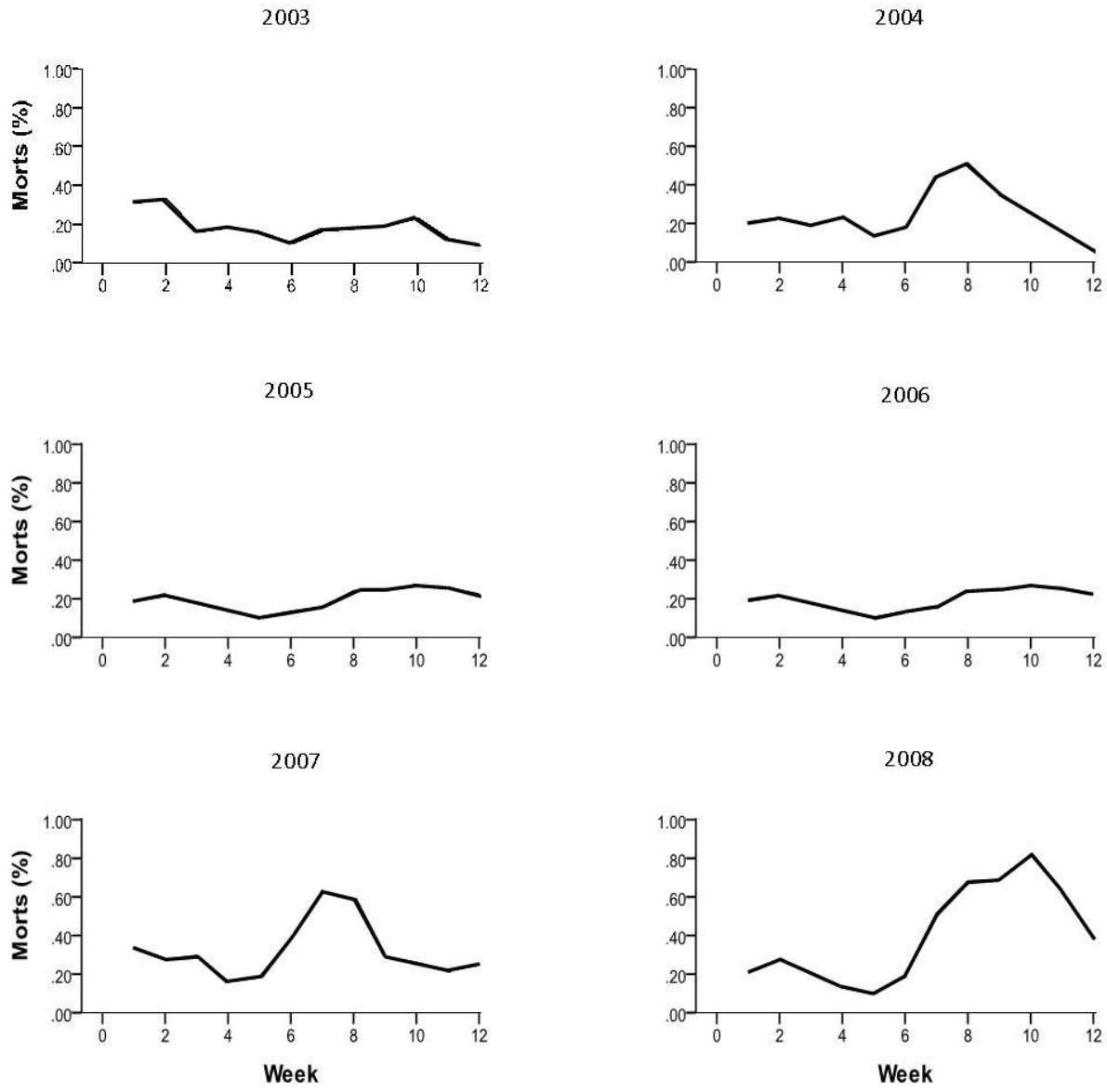


Figure 2.3 Mortality as a percentage of SBT transferred in the first twelve weeks for participating companies combined for 2003 to 2008

2.3.3 Company differences

Figure 2.4 shows the average mortalities as a percentage of SBT transferred for the first 12 weeks post transfer for the four individual companies (Companies 1 to 4) that provided data for at least 5 of the 6 years (2003-2008) of the study. SBT mortalities in Companies 1, 2 and 3 mimic the mortality trend of the whole industry (Figure 2.2) with an elevated mortality rate in the first weeks post transfer followed by a gradual reduction and then a peak in the period 6 to 10 weeks post transfer. Company 4 is somewhat different, displaying a similar initial period of elevated mortality but the later rise is delayed by 2 weeks to week 8 post-transfer and then continues to rise until at least the end of the 12 week study period.

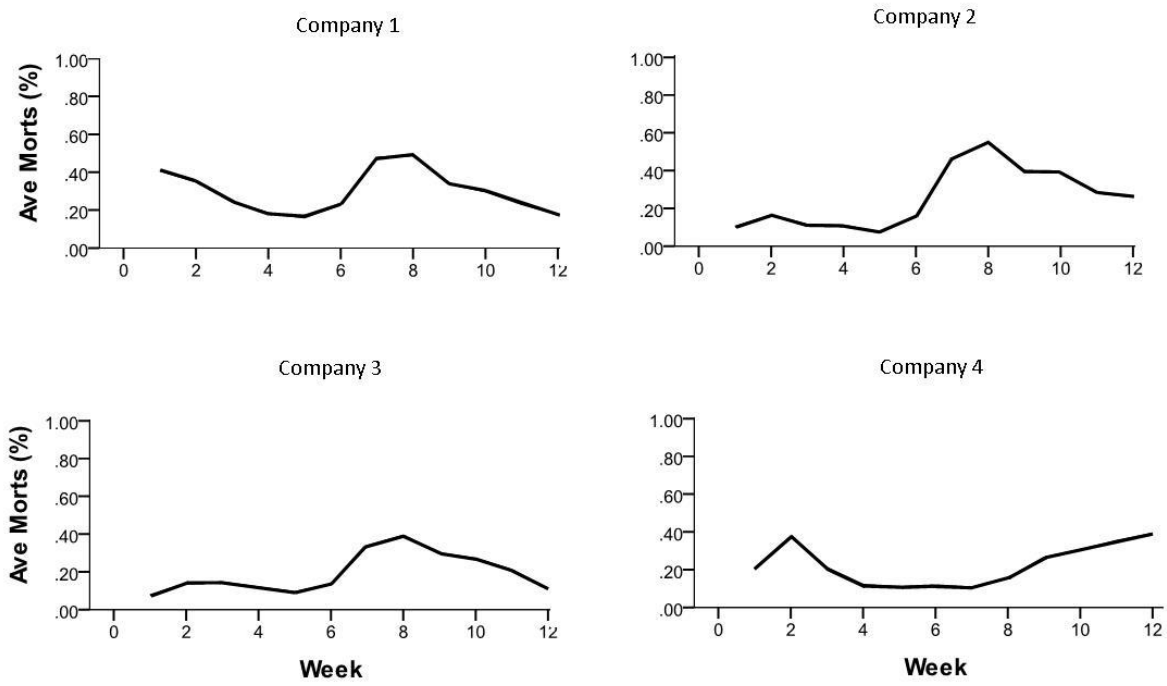


Figure 2.4 Average mortality as a percentage of SBT transferred in the first twelve weeks for each company 2003 to 2008

Figure 2.5 shows the mortalities as a percentage of SBT transferred for four of the companies for the first 12 weeks post transfer in 2008, the last year of the study. Note Company 4 was unable to participate in 2008 and was replaced by another Company (5). Note also the change in the scale of the mortality axis in some of the plots.

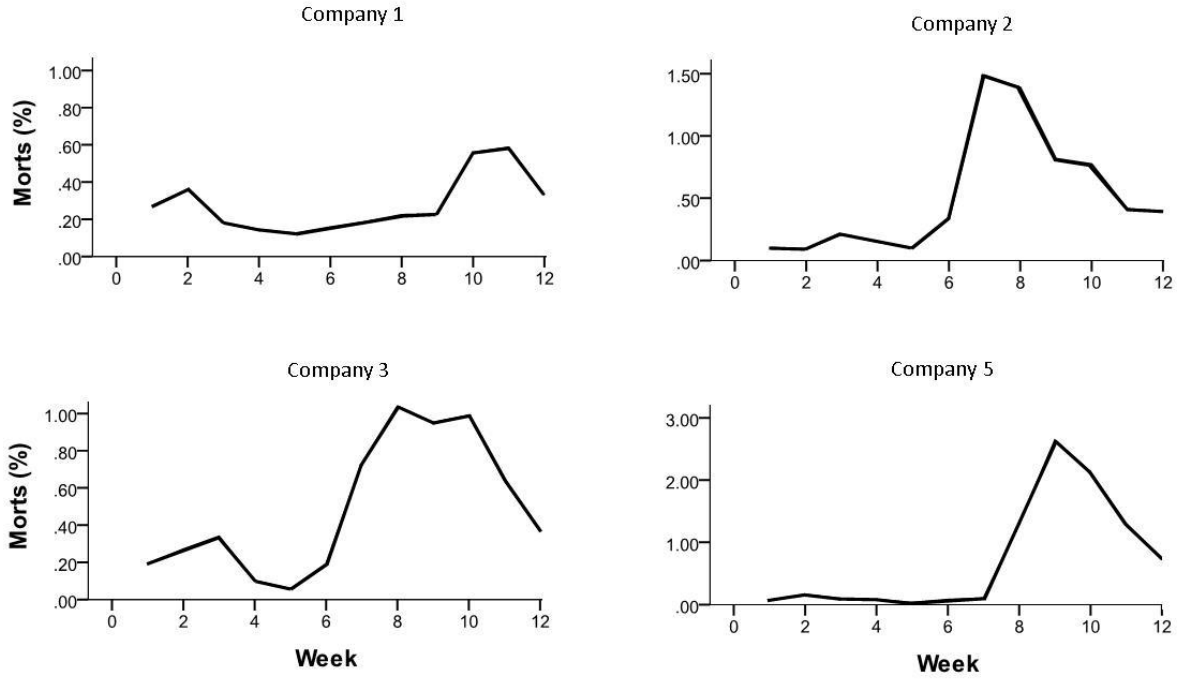


Figure 2.5 Mortality as a percentage of SBT transferred in the first twelve weeks for each company in 2008

By 2008 there was no intercompany difference in the pattern of mortality post transfer, although substantial difference in the scale of mortalities. Mortalities increased markedly 6 to 7 weeks post transfer and remained high for approximately 2 to 3 weeks. Company 1 had considerably reduced mortality over the period studied and Company 5 considerably higher for 2008. An analysis of tow characteristics within the participating companies was of interest in understanding such differences in scales of mortality.

2.3.4 Capture and tow

Section 2.3.1 summarised the whole of industry capture/transport results from 2001 to 2014. The corresponding database for the companies contributing to this study from 2003 to 2008 consisted of 290 transfers into grow-out cages from a total of 73 tow cages over six years, 204 transfers from 50 tow cages for 2003 to 2006 and 86 transfers from 23 tow cages for 2007 to 2008. Table 2.6 and Table 2.7 provide a summary of descriptive statistics for relevant tow variables for these periods respectively. Note the average weight is that sampled at end tow in the then 40 fish hook sample.

Table 2.6 Descriptors of relevant tow variables for 2003 to 2006

2003-2006	Mean	Min	Max
Average weight (kgs)	16.55	11.97	23.53
Tow length (days)	22	12	40
Tow distance (nm)	246	186	314
Tow speed (kts)	0.52	0.28	0.87
Tow density (kg/m^3)	0.38	0.16	0.63
Tow biomass (kg/m^3)	6.15	2.82	10.42
No of fish in tow cage	8476	3153	12801
No of tow mortalities	14	2	49
Transfer time (min)	67	9	218

Table 2.7 Descriptors of relevant tow variables for 2007 and 2008

2007-2008	Mean	Min	Max
Average weight (kgs)	15.96	13.18	22.75
Tow length (days)	26	15	40
Tow distance (nm)	231	195	307
Tow speed (kts)	0.4	0.23	0.69
Tow density (kg/m^3)	0.38	0.09	0.76
Tow biomass (kg/m^3)	5.90	2.06	12.07
No of fish in tow cage	8828	1741	18155
No of tow mortalities	15	2	68
Transfer time (min)	Not recorded	Not recorded	Not recorded

Average weight of the fish captured over the two periods were similar (approximately 0.5 kg difference), density of fish within the tow cages were the same and mortalities recorded during tows were comparable. Tows in 2007 and 2008 were undertaken at a slightly slower average tow speed (0.4 knots compared to 0.52 knots) and therefore on average longer tows (+ 4 days) were undertaken. None of these differences are considered of obvious operational or biological significance in terms of the subsequent performance of the SBT cohorts transported for grow-out.

2.3.5 Company tow experience

Industry average tow data did not assist in investigating increasing mortality trends. A finer scale review of tows at the company level was undertaken. Table 2.8 summarises the tow parameter means for each of the contributing companies. Note only one tow for Company 5 was available for use in the data set.

Table 2.8 Descriptors of relevant tow variables for each company for tows undertaken 2003 to 2008.

	Company				
	1	2	3	4	5
Average weight (kgs)	16.51	15.81	16.60	15.93	17.14
Tow length (days)	21	24	22	30	31
Tow distance (nm)	240	246	242	242	230
Tow speed (kts)	0.51	0.46	0.49	0.35	0.31
Tow density (kg/m^3)	0.38	0.45	0.32	0.34	0.61
Tow biomass (kg/m^3)	6.02	7.01	5.40	5.30	10.49
No of fish in tow cage	8210	10370	7680	8710	12654
No of tow mortalities	10	24	15	25	2

Companies 4 and 5 had slower average tow speeds than the other three and as a result have tows lasting 25 to 45% longer on average. Companies 5 and 2 had higher stocking rates than the other companies.

Table 2.9 is a matrix of Spearman's r_s correlation coefficients for tow variables for 2003 to 2006 for the combined data set. Correlations of note are in red within bold outlined cells.

Table 2.9 Correlation matrix for tow parameters for 2003 to 2006.
Top value in each cell is Spearman's r_s coefficient, bottom value in each cell is the 2-tailed level of probability. Significant correlations are shown in red. *p=0.05*. Values have been rounded to 2 decimal places for ease of presentation.

	Fish Total									
Tow Mortality	-0.21 0.15	Tow Morts								
Density	***0.77*** ***0.00***	-0.21 0.15	Density							
Biomass	***0.58*** ***0.00***	-0.15 0.30	***0.83*** ***0.00***	Biomass						
Length	0.40 0.79	***0.33*** ***0.02***	0.09 0.55	0.05 0.72	Length					
Distance	-0.21 0.89	-0.04 0.80	-0.17 0.32	-0.18 0.24	***0.29*** ***0.05***	Distance				
Speed	-0.04 0.76	***-0.37*** ***0.02***	-0.15 0.31	-0.14 0.34	***-0.93*** ***0.00***	0.05 0.73	Speed			
Morts 1st Week	***0.35*** ***0.01***	-0.18 0.23	***0.44*** ***0.00***	***0.47*** ***0.00***	0.14 0.35	-0.04 0.79	-0.21 0.15	Morts 1st W		
Morts 1st Month	***0.38*** ***0.01***	-0.21 0.16	***0.39*** ***0.01***	***0.43*** ***0.00***	0.17 0.24	0.06 0.15	-0.19 0.19	***0.92*** ***0.00***	Morts 1st M	
Morts 2nd Month	***0.38*** ***0.01***	-0.21 0.15	***0.38*** ***0.01***	***0.41*** ***0.00***	-0.01 0.94	0.17 0.24	0.32 0.83	***0.58*** ***0.00***	***0.66*** ***0.00***	Morts 2nd M
Morts 3rd Month	***0.41*** ***0.00***	-0.12 0.44	***0.42*** ***0.00***	***0.30*** ***0.04***	0.24 0.10	0.15 0.31	-0.20 0.16	***0.70*** ***0.00***	***0.73*** ***0.00***	***0.63*** ***0.00***	Morts 3rd M
Morts 1st 3 mths	***0.42*** ***0.00***	-0.20 0.18	***0.44*** ***0.00***	***0.43*** ***0.00***	0.20 0.17	0.18 0.21	-0.18 0.22	***0.82*** ***0.00***	***0.90*** ***0.00***	***0.87*** ***0.00***	***0.85*** ***0.00***

There was a moderate positive correlation ($0.40 \leq r_s \leq 0.59$) between the number of mortalities throughout the first twelve weeks of grow-out and measures of stocking rate within the tow cages, for example total number of fish, fish density and fish biomass for the period 2003 to 2006. The mortalities on tows had a weak positive correlation ($0.20 \leq r_s \leq 0.39$) with the number of days of the tow and a weak negative correlation with speed of tow (the latter two variables obviously were very strongly negatively correlated).

Table 2.10 is a matrix of correlation coefficients for tow variables for 2007 and 2008. The overall correlations of grow-out mortalities with tow parameters is weaker in the final two years of the study with only a moderate positive correlation ($0.40 \leq r_s \leq 0.59$) of mortality within the second month of grow-out with the extent of crowding (density, biomass) in the tow cage. It appeared additional factors were influencing early grow-out mortalities in 2007 and 2008 than in the earlier years of the study.

Table 2.10 Correlation matrix for tow parameters for 2007 and 2008.
Top value in each cell is Spearman's r_s coefficient, bottom value in each cell is the 2-tailed level of probability. Significant correlations are shown in red. *p=0.05*. Values have been rounded to 2 decimal places for ease of presentation.

	Fish Total										
Tow Mortality	-0.27 0.21	% Tow Morts									
Density	***0.85*** ***0.00***	-0.22 0.30	Density								
Biomass	***0.81*** ***0.00***	-0.14 0.53	***0.96*** ***0.00***	Biomass							
Length	0.11 0.61	-0.16 0.47	0.09 0.70	0.02 0.93	Length						
Distance	-0.37 0.09	0.23 0.30	-0.23 0.31	-0.30 0.18	0.11 0.62	Distances					
Speed	-0.26 0.24	0.23 0.31	-0.16 0.47	-0.15 0.50	***-0.88*** ***0.00***	0.31 0.16	Speed				
Morts 1st week	-0.05 0.84	-0.01 0.96	0.04 0.86	0.06 0.79	-0.38 0.08	-0.23 0.31	0.29 0.19	Morts 1st W			
Morts 1st Month	-0.15 0.50	-0.02 0.94	-0.03 0.91	-0.01 0.95	-0.28 0.20	-0.27 0.22	0.18 0.42	***0.87*** ***0.00***	Morts 1st M		
Morts 2nd Month	0.30 0.17	-0.16 0.46	***0.56*** ***0.01***	***0.50*** ***0.02***	0.09 0.69	-0.19 0.39	-0.13 0.57	0.26 0.24	0.41 0.05	Morts 2nd M	
Morts 3rd Month	-0.18 0.43	0.05 0.81	-0.28 0.20	-0.18 0.41	0.052 0.82	0.01 0.97	-0.14 0.53	-0.11 0.62	-0.05 0.81	-0.24 0.27	Morts 3rd M
Morts 1st 3 mths	0.16 0.47	-0.11 0.63	0.29 0.19	0.32 0.14	-0.04 0.86	-0.19 0.39	-0.07 0.75	0.36 0.10	***0.49*** ***0.02***	***0.66*** ***0.00***	***0.46*** ***0.03***

2.3.6 Grouping of tows

Figure 2.6 displays a cluster analysis grouping of tows undertaken from 2003 to 2006, before any extraneous factors were influencing early grow-out mortalities. A summary of mean values for characteristics common to each grouping is provided in Table 2.11.

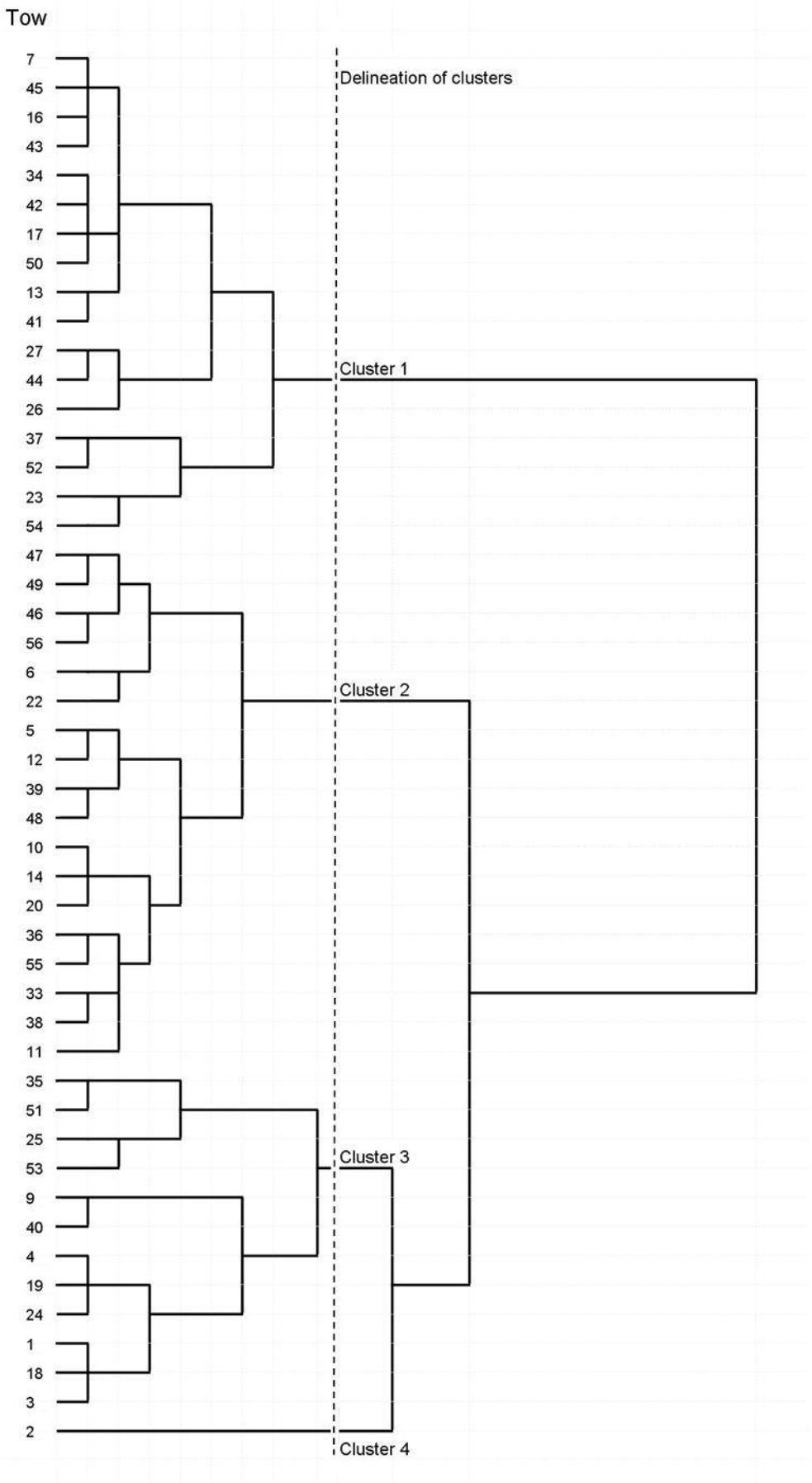


Figure 2.6 Hierarchical cluster of tow groupings for tows from 2003 to 2006

Table 2.11 Characteristics of tow groups for tows from 2003 to 2006
Subjective rankings are L = low, M = medium, H = high, E = extreme

	No of fish	Tow density (fish/m^3)	Tow biomass (kg/m^3)	Tow speed knots	% of Morts after 12 wks
Cluster 1	6391 (L)	0.28 (L)	4.7 (L)	0.54 (M)	1.7 (M)
Cluster 2	9065 (MH)	0.4 (MH)	6.6 (M)	0.54 (M)	1.9 (M)
Cluster 3	9860 (H)	0.46(H)	7.6 (H)	0.43 (L)	4.8 (H)
Cluster 4	10485 (E)	0.36(M)	6.1 (M)	0.87 (H)	10.8 (E)

Tows within clusters 1 and 2 had the lowest mean mortality percentages (< 2%) through the first 12 weeks of grow-out. These tows had the lowest density and biomass of SBT in the tow cages, less than 0.4 fish / m^3 and less than 6.6 kg / m^3 respectively.

2.3.7 Transfer time

Figure 2.7 is a scatter plot of time taken to transfer fish against percentage mortality within each grow-out cage after 12 weeks for the period 2003 to 2006. Regression analysis returned an adjusted R^2 of 0.055 which indicates that overall less than 6% of the variation in mortality subsequent to transfer can be attributed to the time taken to transfer SBT from the tow cage to the grow-out cage. This aspect of the study was discontinued for post 2006 transfers.

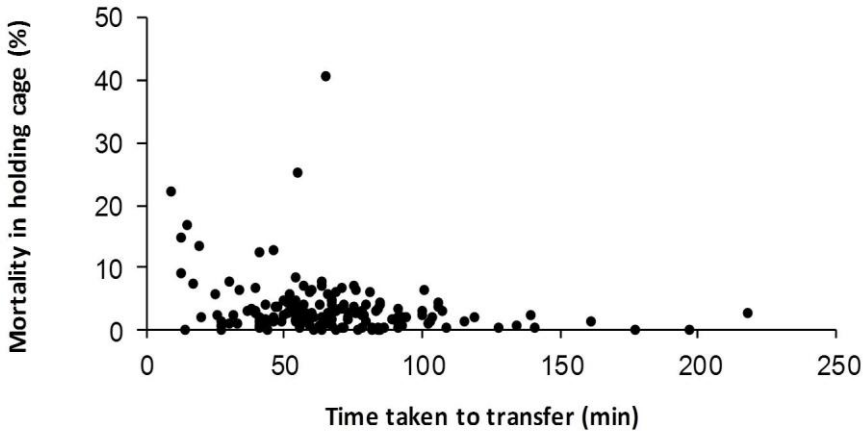


Figure 2.7 Transfer time against percent mortality in holding cage after 12 weeks

2.4 DISCUSSION

The capture and transport stage of SBT ranching appears to have always at least been equal to world's best practice in terms of initial mortality. Capture/transport mortalities of less than 0.5% (0.08% in recent years) are lower than those reported for other capture-based aquaculture sectors (yellowtail 20 to 30%, Nakada 2008; grouper 50% or more, Ottolenghi et al 2004; ABT up to 2%, Katavic et al 2003b; PBT 1 to 3% Zertuche-González et al 2008).

Accepting the premise that the efficacy of the capture and transport stage could contribute to subsequent survival in at least the early grow-out weeks, the performance of the SBT ranching sector from capture is further supported. Total grow-out mortalities recorded for SBT ranching up until 2006 of 2 to 5% were comparable to those during grow-out of yellowtail (2%, Nakada 2008), ABT (3 to 5%, Katavic 2003b) and PBT (6%, Zertuche-González et al 2008).

The low level and relative consistency in the pattern of mortality in the first twelve weeks of SBT grow-out in the early 2000s (Figure 2.3) suggest this period was representative of normal operational loss of stock. In this context, operational losses are considered those that are incurred from the established methodology of purse seine net encirclement, sea transfer, tow to grow-out sites and transfer into grow-out cages. Such losses are unlikely to be avoided unless wholesale changes are made to husbandry practices. Though there have been some attempts to review the capture/transport methodology involved in tuna aquaculture operations (Beaz and Núñez 2003, de Bartolomé and Méndez 2005), there has been little change in the capture and transport technology used for tuna world-wide over the last 15 years and major changes are unlikely for the foreseeable future. Although some refinement in practices has resulted in the reduced pre-transfer SBT mortality rate (Figure 2.1), the lack of major infrastructure innovation suggests that mortalities of 2 to 5% during grow-out of SBT may be considered within the range of predictable operational losses.

By extension, mortalities above predictable operational losses at any time can be considered of extraneous origin and not acceptable within suitably managed SBT operations. Such extraneous influences on mortalities were considered evident in the mid 2000s, and particular from the latter two years of this study (2007, 2008) and on into 2010. It is suggested years 2007 and 2008 (Figure 2.3) showed the first clear influence of an extraneous mortality factor, with normal elevated mortalities in the first two weeks after transfer (conforming to Croatia's higher mortalities during the first month of adaptation after capture, Katavic 2003b), followed

by a reduction back to operational levels followed by a large spike beginning from 6 to 8 weeks and lasting to 9 to 11 weeks before reducing again. The 6 to 11 week spike in mortality was greater in 2008 than 2007 and though not detailed as part of this study, the extraneous factor involved can be seen to have continued to cause increasing mortalities up to 2010, peaking at 13.5% (Figure 2.2). Considerable research has been conducted since the late 2000s to investigate specifically the cause of extraneous mortality in SBT ranching. Infection by the aporocotylid blood flukes *Cardicola forsteri* and *C. orientalis*, a group of parasites that has major impacts on tuna aquaculture operations world-wide (Balli et al in press), was found to be the cause of the mortalities (Aiken et al 2006, Shirakashi et al 2013). Subsequent treatment of SBT with the antihelmintic praziquantel (Hardy-Smith et al 2012, Shirakashi et al 2012) since 2011 has been attributed with the dramatic reduction in mortality to levels of 1.3 and 3.2% in 2013 and 2014 respectively.

Notwithstanding the success of blood fluke treatment, even a difference in mortality of 3.2% compared with 1.3% has economic consequences, and the challenge has remained to investigate ways to improve the capture and transport of SBT to reduce operational losses where possible. Testament to the need of meeting this challenge was the differences in company performance evident in this study. Companies 1, 2 and 3 appeared to have similar and 'industry standard' mortality profiles with higher levels in the two weeks immediately after transfer, followed by a reduction back to operational levels depending on whether blood fluke treatment had been commenced or not. In 2007 and 2008 (and through to 2010) spikes in mortality from about 6 to 11 weeks were most likely from blood fluke infection (Aiken et al 2006). Company 4 however had the equal highest average mortality in the initial two weeks after transfer and exhibited an increasing rate of mortality that was still unchecked by the end of the 12 week period of interest (Figure 2.4). It is suggested that Company 4 had atypical capture/transport, transfer or grow-out husbandry practices that contributed to these elevated mortality profiles. Unfortunately Company 4's withdrawal from the study at the end of 2007 prevented any detailed analysis of this premise.

Quicker/shorter tows in the period 2003 to 2006 resulted in lower mortalities during the towing period (Table 2.9) however mortalities on tow were so low this is of incidental interest. During that period early grow-out mortalities were a function of the density and biomass of SBT in the tow cage (Table 2.9), with higher mortalities throughout the first 12 weeks of SBT coming from higher stocked cages. This correlation was weakened in the results from 2007 and 2008 though it is suggested any underlying mortality trends from operational issues during these years was likely smothered by the apparently increasing influence of blood fluke infection.

The years 2007 and 2008 were useful in investigating any company differences. All the companies (with Company 5 replacing Company 4) in 2008 displayed the then typical mortality pattern with the blood fluke related spike at 6 to 11 (or 12) weeks (Figure 2.5). However the new Company 5's mortality levels were up to 4 times that of some of the other companies, supporting the premise that individual company capture/transport practices may influence subsequent mortalities in early grow-out. Companies 4 and 5 averaged longer tows than the other companies (30 to 31 day compared to 21 to 24 days, Table 2.8). Unfortunately feed regimes on tow were not provided by participating companies so it is unknown if starvation had some influence here. These companies also had amongst the highest number of SBT in their tow cages (Table 2.8).

Grouping like tows together indicated that lower mortalities in grow-out resulted from tow cages with lower density and biomass of SBT (Figure 2.6 and Table 2.11). Tow parameters for optimal grow-out results in terms of mortality from this study were tow densities of less than 0.3 fish /m^3, tow biomass less than 5 kg /m^3 and average tow speeds of around 0.5 knots. It is suggested that tow density is likely the most important of these parameters but this was not definitively tested. There appeared to be little influence of the time within which transfers of SBT into grow-out cages were made on subsequent mortality.

Remembering the fish farmers challenge, over the study period it was evident that some companies obtained better rates of survival through to harvest than others, and that this in part could be related to capture and transport practices. The overall performance of the industry in this stage of its operation as adjudged by the admitted course measure of mortality appears acceptable, and operators match overseas competitors in regards to this aspect of ranching. The study documents for the first time the capture/transport mortality rate of less than 0.5% for the SBT ranching sector and suggests normal operational losses of 2 to 5 % through grow-out. It suggests the now recognised *Cardicola* threat became of economic importance around 2007.

Of direct application to industry are the recommended tow parameters of a density less than 0.3 fish/m^3, a biomass less than 5 kg/m^3 and tow speeds of around 0.5 knots. Restricting the length of time with the fish in the tow cage unfed also is emphasized. To provide a more extended measure of ranching performance, stress and immune response indicators following tow and through the ranching cycle to harvest were investigated.

CHAPTER 3 STRESS AND IMMUNE RESPONSES OF RANCHED SBT

3.1 INTRODUCTION

Avoidance of stressors in their environment is not an option for SBT following capture. As evident in the preceding discussion, the challenges of a substantial period of crowding during towing followed by forced activity during transfer and an extended grow-out period must be met. To do so the cascade of physiological processes and accompanying stress and humoral immune responses outlined in Section 1.5 is available in an attempt to maintain a suitable homeostatic state. These responses have been studied considerably in other fish species (Table 1.4) and are accepted as providing an insight into health status. A principal objective was to establish which indicators were of the highest utility in this regard for SBT and to investigate any seasonal or annual differences. Health indicators can also provide an indication of how effective husbandry practices may be suited to a species (Iwama et al 1997, Ellis et al 2012), which is of obvious interest for SBT ranchers. Much of the literature on stress responses comes from cultured stocks and the value of determining base responses in wild fish populations has been emphasised (Pankhurst 2011). The physical restraint and sampling methodology required in measuring these responses in a large, pelagic fish has limited the body of such research undertaken with SBT and bluefin tuna in general (Table 3.1) and another objective was to establish base levels of health indicators in wild SBT.

Table 3.1 Stress and immune response indicators in wild bluefin tuna
[A] range from several years study during the European Union REPRO-DOTT program, [B] converted from mg/dL glucose where mg/dL x 0.055 = mmol/L, [C] range from 3 years sampling 2010 to 2012, [*]approximate values transposed from graphical presentation

Indicator	Mean/Range	Species	Source
Cortisol (ng/mL)	6 to 600[A]*	ABT	Anon 2007
	73 ± 9.5	ABT	Addis et al 2013
Glucose (mmol/L)	5.7 ± 0.8[B]	ABT	Percin and Konyalioglu 2008
	14.0 ± 1.6[B]	ABT	Mišlov Jelavić et al 2012
	4.6 ± 0.4[B]	ABT	Addis et al 2013
	5.8 to 7.4[C]	SBT	Kirchhoff et al 2014
Lactate (mmol/L)	17.0 ± 2.2	ABT	Addis et al 2013
	6.9 to 10[C]	SBT	Kirchhoff et al 2014
Lysozyme (µg HEWL/mL)	20 to 28*	SBT	Watts et al 2002
	9.4 to 50[C]	SBT	Kirchhoff et al 2014
Haemoglobin (g/dL)	13 to 18	SBT	Rough et al 2005
	22 to 28[C]	SBT	Kirchhoff et al 2014
pH	7.8 to 8.14[C]	SBT	Kirchhoff et al 2014
Osmolality (mOsmol/kg)	401 to 440[C]	SBT	Kirchhoff et al 2014
IgM (mg/mL)	0.7 to 1.3*	SBT	Watts et al 2002
ACH50 (units/mL)	3 to 7*	SBT	Watts et al 2002
	75 to 253[C]	SBT	Kirchhoff et al 2014
CH50 (units/mL)	9 to 43*	SBT	Watts et al 2002

A considerable range is evident in most of the indicators particularly cortisol, glucose, lactate and components of the complement pathways (CH50 and ACH50). Part of this variation likely is due to handling variations at the time of sampling. The wild fish glucose results reported by Mišlov Jelavić et al (2012) are considerably higher than the other studies listed in Table 3.1 and were elevated to similar levels when sampled after six months of farming suggesting a sampling effect within that study. Similarly, the very disparate range in cortisol levels reported for wild ABT throughout the REPRO-DOTT program (Anon 2007) came from fish caught using drift nets, handline, purse seiner and set net traps, all of which involve considerable delay/activity. It is likely the highest range of values for cortisol, glucose and lactate in Table 3.1 result from sampling subsequent to the initiation of a stress handling response. There is also considerable variation in the alternative complement activity (ACH50) of SBT reported by Kirchhoff et al (2014). That study indicated inter-annual differences in blood variables and even differences within schools of SBT within the same year.

As noted in Section 1.5 the time interval and methods of handling/sampling fish for blood analyses are important, particularly where catecholamines and corticosteroids (ie cortisol) are of interest (Ellis et al 2012). For example the REPRO-DOTT capture methods mentioned above resulted in a wide range of cortisol measures of 15 to 160 ng/mL for drift nets, 6 to 360 ng/mL for handlines, 20 to 90 ng/mL for purse seine nets and 15 to 180 ng/mL for set net traps (Anon 2007). Given the usually larger size of ABT it is likely that all of the above techniques, including purse seine, involved at least many minutes of the fish struggling or under forced activity before blood could be sampled. In contrast the smaller SBT in this study and most of those for the SBT results reported in Table 3.1 were caught either by pole and line, baited hook on a heavy trace or by diver and were euthanised and sampled within 30 seconds to 2 minutes.

3.1.1 Captive bluefin stress and immune responses

For the purpose of subsequent discussion four stages will be recognised in bluefin tuna capture-based aquaculture, pre-capture or wild, end tow, mid season and harvest. End tow subsumes all of the influences of capture, transfer into the tow net and the extended period under towing conditions (occasionally 7 weeks, Marnikol Fisheries pers comm) up to immediately before transfer in to grow-out nets. The logistical difficulties of sampling on the open water of fishing grounds during or soon after capture, the debatable value of intermediate sampling points during tows and the high individual value of fish often needed to be sacrificed during sampling have prevented extensive measuring of blood indicators between the wild and

end tow stages. Harvest represents the obvious end point of the time held in captivity and mid season refers to an approximate period midway between transfer and harvest.

Most of the studies cited in Table 3.1 went on to include some aspects of bluefin tuna held in captivity. Table 3.2 provides a summary of the stress and immune response indicators measured under captive conditions. Corresponding stages of captivity as described above have been allocated where appropriate to allow some subsequent comparisons.

Table 3.2 Stress and immune response indicators in captive bluefin tuna. Unless otherwise cited the source for subsequent captive stage values is the same as that for the preceding stage. The mid season stage includes a number of different weeks in captivity indicated in (brackets). [A] converted from mg/dL glucose where mg/dL x 0.055 = mmol/L, [B] Total Ig reported, * approximate values transposed from graphical presentation, na described only as captive fish hooked from cage

Indicator	Mean/Range	Species	Captive stage	Source
Cortisol (ng/mL)	4.74 ± 2.18	SBT	Mid season (6)	Thomas et al 2003
	148 ± 21.2	ABT	Exhaustive capture	Addis et al 2013
Glucose (mmol/L)	11.2 ± 0.29[A]	ABT	Mid season (na)	Percin and Konyalioglu 2008
	3.03 ± 0.29	SBT	End tow	Kirchhoff et al 2011a
	5.79 ± 0.35	SBT	Mid season (6)	
	6.22 ± 0.16	SBT	Harvest	
	6.9± 0.65	ABT	Harvest	Topić Popović et al 2012
	14.6 ± 2.9[A]	ABT	Harvest	Mišlov Jelavić et al 2012
	7.14 ± 1.07[A]	ABT	Exhaustive capture	Addis et al 2013
Lactate (mmol/L)	2.02 ± 0.31	SBT	Mid season (6)	Thomas et al 2003
	10.16 ± 0.29	SBT	End tow	Kirchhoff et al 2011a
	11.09 ± 0.39	SBT	Mid season (6)	
	9.59 ± 0.29	SBT	Harvest	
	17.0± 0.22	ABT	Exhaustive capture	Addis et al 2013
Lysozyme (µg HEWL/mL)	71.69 ± 27.56	SBT	End tow	Kirchhoff et al 2011a
	151.03 ± 27.56	SBT	Mid season (6)	
	73.04 ± 14.54	SBT	Harvest	
	25.43 ± 8.99	SBT	End tow	Kirchhoff et al 2012
	171.88 ± 17.34	SBT	Mid season (8)	
	33.49 ± 4.81	SBT	Harvest	
Haemoglobin (g/dL)	15.3 ± 0.8	SBT	Mid season (4)	Clark et al 2008
	19.4 ± 0.5	SBT	End tow	Kirchhoff et al 2011a
	20.33 ± 0.37	SBT	Mid season (6)	
	18.84 ± 0.27	SBT	Harvest	
pH	7.87 ± 0.05	SBT	End tow	Kirchhoff et al 2011a
	8.09 ± 0.03	SBT	Mid season (6)	
	7.66 ± 0.03	SBT	Harvest	
Osmolality (mOsmol/kg)	394.2 ± 4.5	SBT	End tow	Kirchhoff et al 2011a
	412.6 ± 4.5	SBT	Mid season (6)	
	451.5 ± 4.6	SBT	Harvest	
Ig (mg/mL)	1.5[B]*	SBT	Mid season (12)	Watts et al 2002
	3.6 to 5.2[B]*	SBT	Harvest	
ACH50 (units/mL)	5*	SBT	Mid season (10)	Watts et al 2002
	42 to 50*	SBT	Harvest	
	184.38 ± 23.81	SBT	End tow	Kirchhoff et al 2011a
	119.56 ± 10.3	SBT	Mid season (6)	
	85.28 ± 6.08	SBT	Harvest	
	156.40 ± 18.63	SBT	End tow	Kirchhoff et al 2012
	115.2 ± 12.3	SBT	Mid season (8)	
	48.2 ± 4.1	SBT	Harvest	
CH50 (units/mL)	7*	SBT	Mid season (12)	Watts et al 2002
	68 to 93*	SBT	Harvest	

3.2 METHODS

Blood variables in three cohorts of ranched SBT were investigated over three years 2006, 2007 and 2008. Contributing companies were assigned a unique tow cage identifier (T1 to T6) for each of their tow cages from which SBT were sourced. Hence T1 samples in 2006, 2007 and 2008 all came from the same company throughout the study. The first year's pilot survey (2006) included SBT sampled from three tow cages (T1, T4 and T6). Sampling in subsequent years followed five tow cages in 2007 (T1, T2, T3, T4 and T5) and four tow cages in 2008 (T1, T2, T4 and T5). Preferably SBT from each cage were sampled from 4 ranching stages; **wild** populations before the start of fish capture, at the average weight sampling for quota monitoring at **end tow**, **mid season** after 3 or 4 months in grow-out cages and at **harvest**. Due to weather restrictions and/or company confidentiality not all tow cages could have wild fish and/or all of the preferred ranching stages sampled. For ease of discussion each combination of tow/ranching stage is hereafter simply referred to as a group. Table 3.3 is a summary of the sampling undertaken over the study and shows a total of 41 groups from 6 tows from 3 cohorts.

Table 3.3 Sample sizes for SBT groups sampled for blood variables in the 2006, 2007 and 2008 cohorts.

Year	Ranching stage	Tow					
		1	2	3	4	5	6
2006	Wild	10					10
	End tow	5			10		10
	Harvest	10			10		10
2007	Wild	10	10	10	8	5	
	End tow	10	10	10	10	10	
	Mid season	10		10	10	10	
	Harvest	10		10	10	10	
2008	Wild	10	10		10		
	End tow	9	10		10	10	
	Mid season	10	10		9	10	
	Harvest	10	10		9	10	

3.2.1 Blood sampling

The number of fish sampled on each occasion is shown in Table 3.3. All wild fish and the fish sampled at the end tow and mid season were caught on a hand line with a barbless hook. Harvest fish were caught by diver. Fish caught by a hand line were sampled from the lateral

artery posterior to the pectoral fin either by a needle and syringe or from a bleed cut immediately after euthanasia if destructively sampled. All euthanised fish were killed by spiking to the brain (*ike jime* method) then immediately sampled from a bleed cut to the lateral artery. Harvest fish were further processed by coring into and wire pithing of the central spinal cord to disrupt post mortem lactic acid build up and the gills and viscera excised. Blood samples were held on ice until back at the laboratory.

3.2.2 Analytical methods

Blood samples were left overnight at 4 ^{o} C to clot and the serum then separated and frozen. Long-term storage was undertaken at -80^{o} C. Standard laboratory protocols were used to measure the stress and immune response indicators. Cortisol was determined by radioimmunoassay (RIA) using an ethyl acetate extraction step following Pankhurst & Sharples (1992). It has a detection limit of 0.6 ng/mL. Three samples in 2006 had cortisol levels below this detection limit and 0.6 ng/mL was substituted for these samples in subsequent statistical analyses. Specific laboratory instruments were used as per manufacturer's instructions to determine the concentrations of lactate and glucose (Analox GM7 Analyser; Series II), pH (Minilab Isfet pH meter Model IQ125, IQ Scientific, USA) and osmolality (Vapro© Model 5520 vapour pressure osmometer, Wescor Inc., USA). Photometric techniques were used to determine concentrations of haemoglobin (Cobas Mira reading at 540 nm following Drabkin's cyanomethaemoglobin method) and lysozyme (Titertek Multiscan MC plate reader following lysis of suspensions of the bacterium *Micrococcus leisodeikticus*). IgM and IGF were quantified using ELISA analyses. A capture-ELISA following Watts et al (2002) was used to measure IgM. Tuna serum and Ig standards were transferred to capture-antibody coated microtitre well plates, incubated and rabbit antiserum to Tuna Ig added. The complex was detected with alkaline phosphatase-conjugated goat anti-serum to rabbit IgG. An appropriate substrate was added, absorbance read at 405 nm (Titertek Multiscan MC plate reader), and sample IgM levels calculated with reference to standard curves. IGF was quantified utilising a commercially available RIA kit (GroPep Ltd., Thebarton, Australia). The kit uses recombinant tuna IGF-I as the standard and an antibody raised in rabbits against recombinant barramundi IGF-I as antigen as developed by Dyer et al. (2004). Recombinant tuna IGF-I was prepared using a chloramine-T iodination method, and standards, samples and anti-barramundi IGF-I polyclonal antiserum all diluted in a radioimmunoassay buffer. Following separation of bound and free tracer the precipitate was centrifuged, aspirated and bound radioactivity measured with a γ counter.

3.2.3 Statistical inference

Unfortunately studies of tuna ranching inherently are susceptible to contravening some of the assumptions underlying the application of standard statistical techniques. Tuna are large, difficult to handle, expensive fish. Equality of variances amongst experimental groups is an important assumption of analysis of variance (ANOVA), yet the challenges in experimental manipulation of tuna usually meant large sample sizes were not available to maintain variability of data sets within preferred bounds. Equal sample sizes likewise are recommended for the use of most standard post hoc tests (ie Tukey HSD and Student Newman–Keuls). However the difficulties in sometimes extracting blood samples non-destructively from such powerful fish, or laboratory mishaps, and the inability to resample often provided unbalanced sample sets. Non parametric tests (ie Kruskal-Wallis) make fewer assumptions but are less powerful (i.e. less likely to detect real differences as being statistically significant).

The approach adopted in this study for the humoral indicators was to use ANOVA with a conservative decision making probability of $\alpha = 0.01$ rather than 0.05 for statistical significance. This offsets the tendency to be unknowingly testing at a higher than desired probability level due to the skewing of the F-distribution with unequal variances. Post hoc testing of multiple comparisons between means used the Games-Howell procedure which allows for heterogenous sample variances as well as unequal sample sizes. Games-Howell achieve this elegant solution by replacing the standard error of the form commonly used in post hoc testing,

$$\sqrt{\frac{2MS_{error}}{n}} or \sqrt{\frac{MS_{error}}{n}}$$

with a standard error allowing for differing variances and sample sizes.

$$\sqrt{\frac{\frac{s_i^2}{n_i}+\frac{s_j^2}{n_j}}{2}}$$

The corresponding degrees of freedom are similarly adjusted.

$$df' = \frac{\left(\frac{s_i^2}{n_i}+\frac{s_j^2}{n_j}\right)^2}{\frac{\left(\frac{s_i^2}{n_i}\right)^2}{n_i-1}+\frac{\left(\frac{s_j^2}{n_j}\right)^2}{n_j-1}}$$

In undertaking post hoc significance testing, means were ranked in ascending order and the lowest mean compared to the next highest to test if significantly different at a probability level of $\alpha = 0.01$. If not, the lowest mean was then compared to the next mean in order and so on until a significant comparison was evident. No further comparisons of means greater than the first significantly different mean was undertaken. Pair-wise comparisons were then made progressively with the second lowest mean and so on until all possible multiple comparisons were considered. Comparisons of each indicator were made for each sampled group for each of the ranching stages of the three years of the study to investigate seasonal differences. For the inter-annual comparisons data from like groups of each of the ranching stages from each year of the study were pooled to investigate potential differences between years. Testing was as described above; one way ANOVA with Games Howell poc hoc comparisons, using a probability level of $\alpha = 0.01$ for statistical significance. Finally, Spearman's r_s coefficient was calculated for each of the pairwise comparisons of the indicators of individual SBT sampled for all three years of the study. All statistical inference testing was undertaken using SPSS software.

3.3 RESULTS

The present study includes the most extensive investigation of the stress and immune response measures of wild SBT. As noted in Section 3.1, there is intrinsic value in collating and presenting this data separately (Table 3.4). Subsequent sections begin with the range of means $\pm$ the standard error of the mean for the indicator of interest before considering individual comparisons.

3.3.1 2006 indicators

Cortisol and glucose concentrations are shown in Figure 3.1 for the initial pilot study of 2006. There was a considerable variation in cortisol levels (8.4 $\pm$ 3.6 to 217.9 $\pm$ 106.4 ng/mL) both between groups of SBT sampled and within each group sampled. No significant difference was evident, however this is due largely to the extreme variation in end tow groups from T4 and T6. Reasons for this variation are considered in Section 3.4. The two wild groups had the lowest cortisol levels. Glucose ranged from 5.4 $\pm$ 0.4 to 7.4 $\pm$ 0.6 mmol/L and there was no significant difference in glucose levels in any of the groups sampled.

Table 3.4 Stress and immune response indicators in wild SBT sampled in 2006, 2007 and 2008
Values are rounded to nearest single decimal place with the exception of some pH measures
NS No sample available

Indicator	Year	Cohort	Mean ± SE	Indicator	Year	Cohort	Mean ± SE
Cortisol	2006	T1	8.4 ± 3.6	pH	2006	T1	8.1 ± 0.1
(ng/mL)		T6	19.0 ± 4.7			T6	7.9 ± 0.1
	2007	T1	76.6 ± 24.4		2007	T1	8.0 ± 0.04
		T2	135.3 ± 38.3			T2	8.1 ± 0.1
		T3	30.5 ± 9.3			T3	7.9 ± 0.4
		T4	264.4 ± 146.9			T4	8.0 ± 0.1
		T5	70.4 ± 24.2			T5	7.5 ± 0.04
	2008	T1	11.1 ± 2.8		2008	T1	8.2 ± 0.1
		T2	8.1 ± 2.5			T2	8.4 ± 0.1
		T4	97.9 ± 12.2			T4	8.7 ± 0.02
Glucose	2006	T1	5.4 ± 0.4	Osmolality	2006	T1	382.6 ± 14.7
(mmol/L)		T6	6.0 ± 0.2	(mOsmol/kg)		T6	377.1 ± 4.8
	2007	T1	5.3 ± 0.3		2007	T1	375.3 ± 6.6
		T2	4.9 ± 0.2			T2	399.9 ± 7.5
		T3	4.7 ± 0.3			T3	430.2 ± 8.5
		T4	7.0 ± 0.7			T4	457.1 ± 20.3
		T5	10.1 ± 1.4			T5	464.0 ± 2.1
	2008	T1	9.5 ± 0.8		2008	T1	441.6 ± 8.5
		T2	7.4 ± 0.3			T2	424.4 ± 3.1
		T4	8.0 ± 0.2			T4	421.6 ± 1.7
Lactate	2006	T1	7.0 ± 1.2	IgM	2006	T1	5.6 ± 1.8
(mmol/L)		T6	1.2 ± 0.2	(mg/mL)		T6	6.8 ± 1.2
	2007	T1	3.6 ± 0.6		2007	T1	12.6 ± 4.5
		T2	1.7 ± 0.3			T2	16.4 ± 8.6
		T3	3.9 ± 0.6			T3	10.1 ± 1.0
		T4	7.3 ± 1.1			T4	7.5 ± 2.1
		T5	10.9 ± 1.1			T5	3.7 ± 2.2
	2008	T1	5.9 ± 1.3		2008	T1	NS
		T2	2.2 ± 0.3			T2	NS
		T4	2.1 ± 0.2			T4	NS
Lysozyme	2006	T1	5.3 ± 1.6	IGF	2006	T1	19.2 ± 3.3
(µg HEWL/mL)		T6	10.5 ± 1.5	(ng/mL)		T6	12.5 ± 1.2
	2007	T1	16.5 ± 7.9		2007	T1	40.1 ± 2.0
		T2	18.8 ± 8.8			T2	30.0 ± 2.3
		T3	18.0 ± 4.8			T3	NS
		T4	17.0 ± 7.1			T4	NS
		T5	18.2 ± 4.1			T5	NS
	2008	T1	11.5 ± 4.7		2008	T1	55.6 ± 3.2
		T2	17.6 ± 4.5			T2	33.3 ± 1.5
		T4	37.9 ± 6.4			T4	24.2 ± 1.2
Haemoglobin	2006	T1	17.9 ± 1.4				
(g/dL)		T6	13.3 ± 1.3				
	2007	T1	18.5 ± 1.0				
		T2	17.4 ± 1.0				
		T3	18.1 ± 0.6				
		T4	18.5 ± 1.2				
		T5	19.5 ± 1.5				
	2008	T1	19.0 ± 0.5				
		T2	16.3 ± 1.4				
		T4	21.1 ± 0.8				

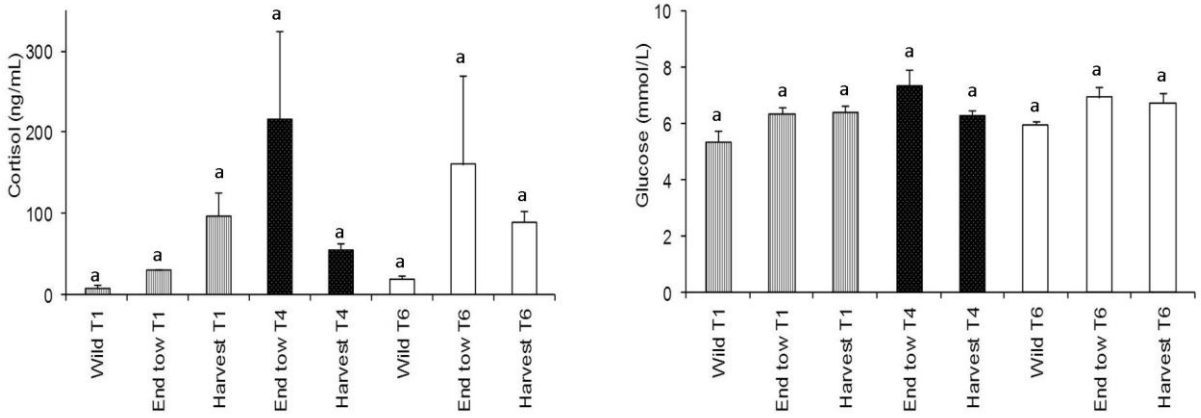


Figure 3.1 2006 cortisol and glucose levels in SBT from various tows (T1, T4, T6) sampled at seasonal ranching stages (wild, end, tow harvest). Error bars show standard error of mean. Labels group means that do not differ *p=0.01*

Lactate and pH levels for 2006 are shown in Figure 3.2. Lactate ranged from 1.2 ± 0.2 to 15.0 ± 1.3 mmol/L, with the latter value from T6 harvest being significantly higher than all the others with the exception of that of T1 harvest. The elevated T1 wild lactate level is possibly sampling related as two of the three SBT with the highest lactate levels also had higher than the average cortisol levels suggesting a handling stress response had initiated. The pH levels ranged from 7.9 ± 0.1 to 8.4 ± 0.1 and there was no significant difference in the pH levels of SBT sampled in 2006.

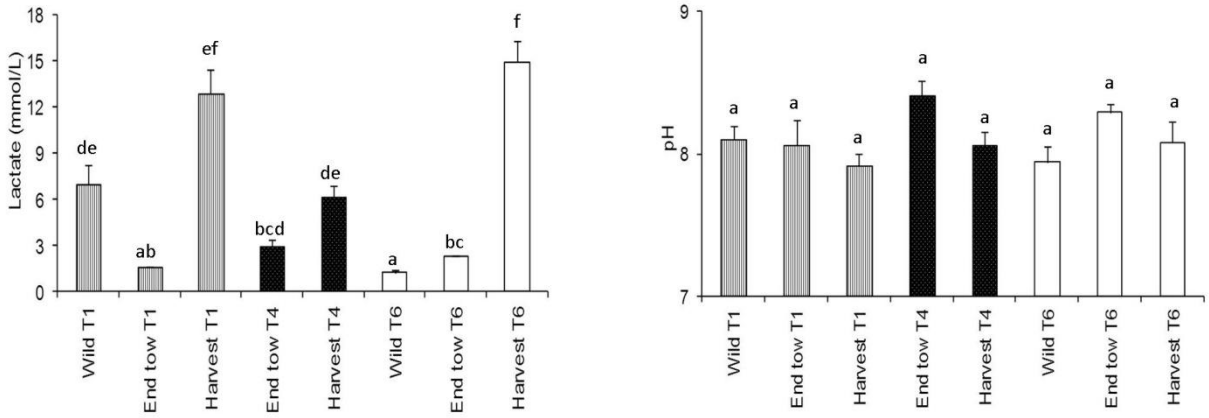


Figure 3.2 2006 lactate and pH levels in SBT from various tows (T1, T4, T6) sampled at seasonal ranching stages (wild, end tow, harvest). Error bars show standard error of mean. Labels group means that do not differ *p=0.01*

Lysozyme and IgM levels are shown in Figure 3.3. Lysozyme ranged from 5.3 ± 1.6 to 64.8 ± 8.5 µg HEWL/mL, and in two of the harvest groups (T4 and T6) levels were significantly higher than in the wild fish. IgM ranged from 5.5 ± 2.4 to 9.8 ± 2.4 mg/mL and there was no significant difference in IgM levels in the groups of SBT sampled in 2006.

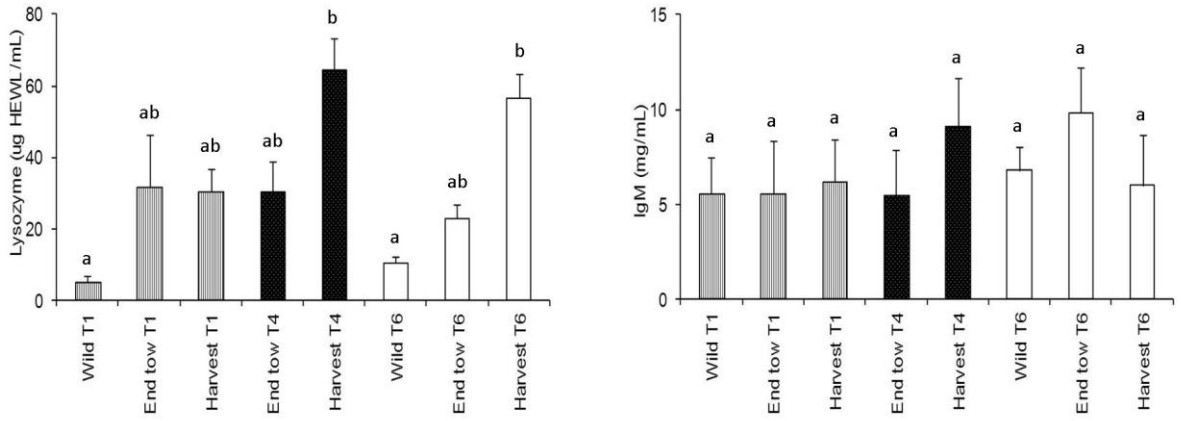


Figure 3.3 2006 lysozyme and IgM levels in SBT from various tows (T1, T4, T6) sampled at seasonal ranching stages (wild, end tow, harvest). Error bars show standard error of mean. Labels group means that do not differ *p=0.01*

Blood osmolality and haemoglobin levels are shown in Figure 3.4. Osmolality ranged from 369.8 $\pm$ 9.2 to 440.9 $\pm$ 13.2 mOsmol/kg and in one of the tows (T6) was significantly higher at harvest than in the wild fish or at end tow. Though osmolality was highest in all harvest groups no other significant differences were evident. Haemoglobin ranged from 12.4 $\pm$ 2.1 to 18.9 $\pm$ 1.3 g/dL and there was no significant difference in Hb levels in the groups of SBT sampled in 2006.

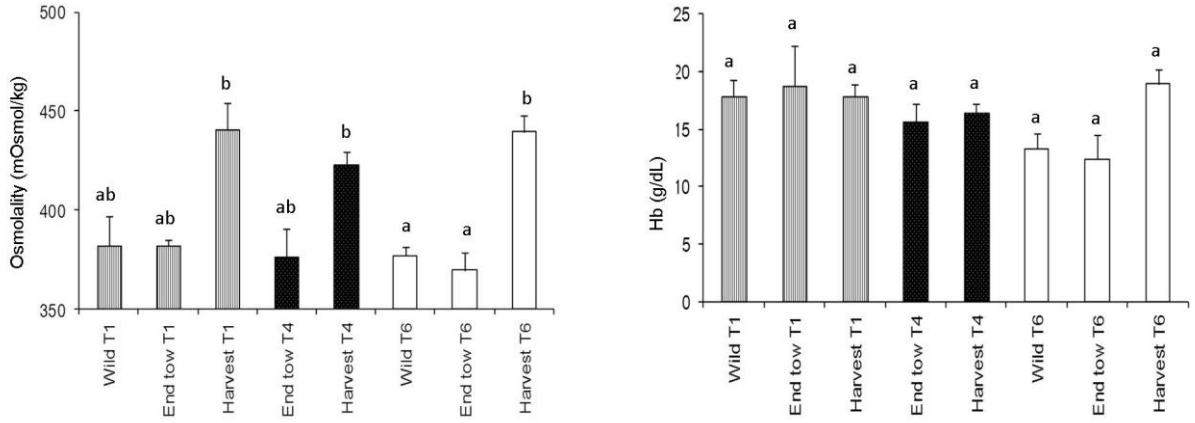


Figure 3.4 2006 osmolality and Hb levels in SBT from various tows (T1, T4, T6) sampled at seasonal ranching stages (wild, end tow, harvest). Error bars show standard error of mean. Labels group means that do not differ *p=0.01*

IGF levels for the 2006 groups sampled are shown in Figure 3.5. Levels ranged from 12.5 $\pm$ 1.2 to 19.2 $\pm$ 3.3 ng/mL, and there were no significant differences in IGF levels of sampled SBT.

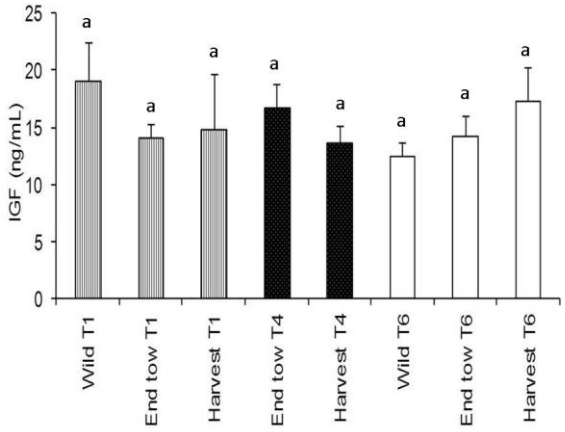


Figure 3.5 2006 IGF levels in SBT from various tows (T1, T4, T6) sampled at seasonal ranching stages (wild, end tow, harvest). Error bars show standard error of mean. Labels group means that do not differ *p=0.01*

3.3.2 2007 indicators

Cortisol and glucose concentrations for 2007 are shown in Figure 3.6. Cortisol ranged from 30.5 $\pm$ 9.3 to 446.8 $\pm$ 86.7 ng/mL and wide variation again was evident in levels between and within groups of fish sampled. Tow 4 had consistently high cortisol levels over the 4 stages sampled (wild, end tow, mid season and harvest) and these were significantly higher than some of the stages sampled in cohorts T3 and T5. Tow 1 had intermediate levels of cortisol not significantly different from the SBT sampled in other tows. Glucose levels ranged from 4.6 $\pm$ 0.4 to 10.1 $\pm$ 1.4 mmol/L and again were not significantly different between groups of SBT sampled in 2007.

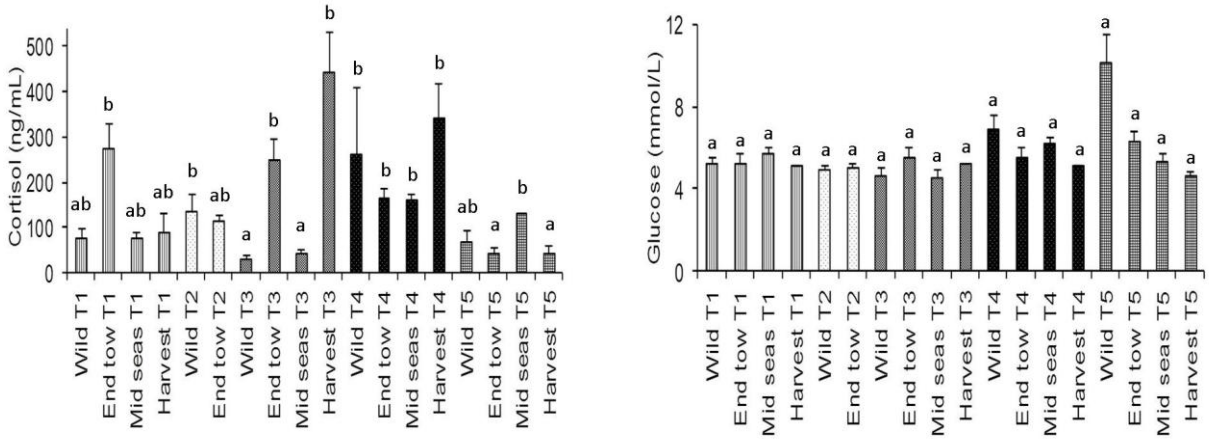


Figure 3.6 2007 cortisol and glucose levels in SBT from various tows (T1, T2, T3, T4, T5) sampled at seasonal ranching stages (wild, end tow, mid seas[on], harvest). Error bars show standard error of mean. Labels group means that do not differ *p=0.01*

Lactate and pH levels in groups sampled in 2007 are shown in Figure 3.7. Lactate ranged from 1.0 $\pm$ 0.1 to 11.0 $\pm$ 1.1 mmol/L, and no consistent pattern was evident. Harvest fish in T3 and wild fish in T5 had significantly higher lactate levels than any other cohort sampled with the exception of T4. The pH levels ranged from 7.50 $\pm$ 0.04 to 8.50 $\pm$ 0.02 and only these two extremes for the T5 wild group and the T4 end tow group respectively were significantly different from some of the other groups.

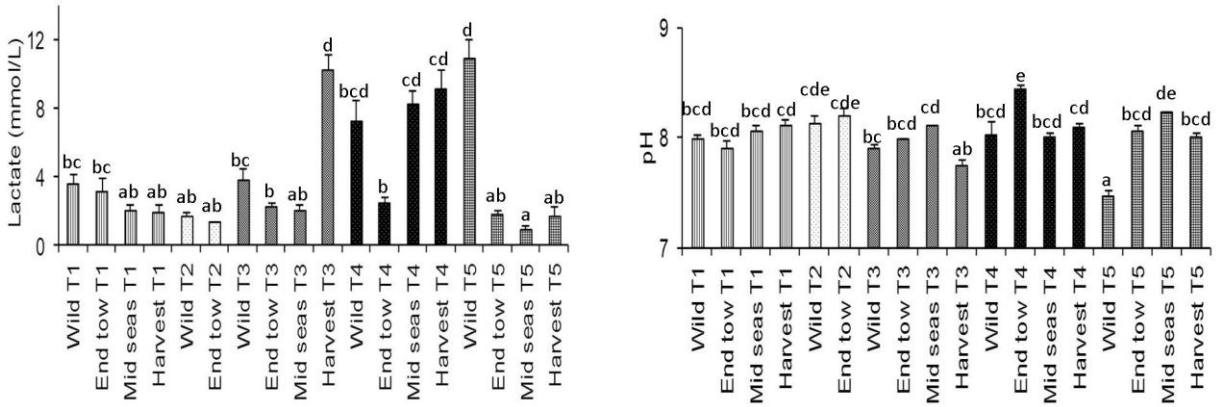


Figure 3.7 2007 lactate and pH levels in SBT from various tows (T1, T2, T3, T4, T5) sampled at seasonal ranching stages (wild, end tow, mid seas[on], harvest). Error bars show standard error of mean. Labels group means that do not differ *p=0.01*

Lysozyme and IgM levels for groups sampled in 2007 are shown in Figure 3.8. Lysozyme ranged from 16.5 $\pm$ 7.9 to 101.3 $\pm$ 6.8 µg HEWL/mL and mid season lysozyme levels were significantly higher than those in wild and end tow SBT in two of the four tows sampled (T1 and T3).

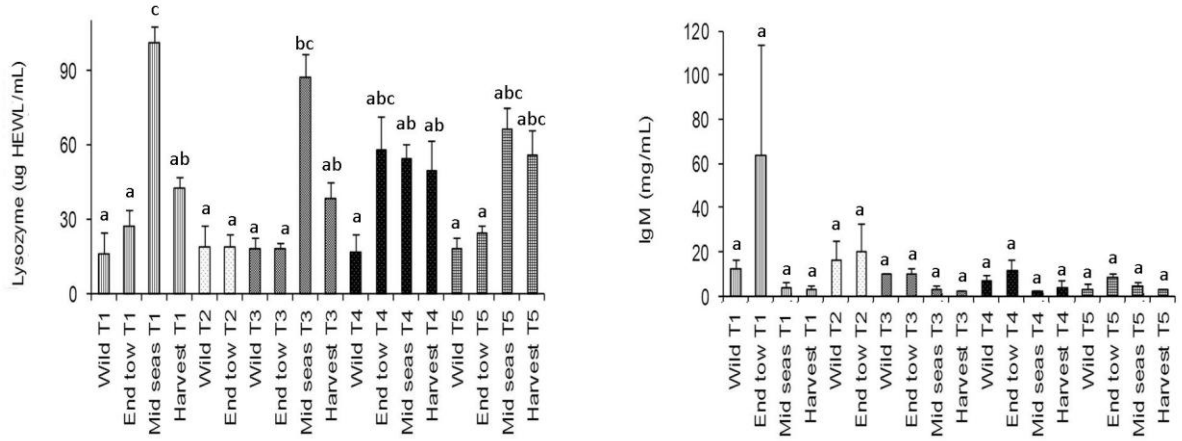


Figure 3.8 2007 lysozyme and IgM levels in SBT from various tows (T1, T2, T3, T4, T5) sampled at seasonal ranching stages (wild, end tow, mid seas[on], harvest). Error bars show standard error of mean. Labels group means that do not differ *p=0.01*

IgM ranged from 2.4 ± 0.5 to 64.5 ± 49.3 mg/mL. The very high variation associated with this latter mean value (wild T1) was due to two extreme results of 97.8 mg/mL and 500 mg/mL for SBT in this group which influenced the testing of significance. Notwithstanding the influence of these two extremes, in all tows of the 2007 cohort the wild and end tow groups of SBT had higher IgM levels than groups from later in the ranching season.

Osmolality and haemoglobin levels for groups sampled in 2007 are shown in Figure 3.9. Osmolality ranged from 375.3 ± 6.6 to 464 ± 2.1 mOsmol/kg. Wild T1 fish had significantly lower osmolality than the wild T3, wild T4 and wild T5 groups and the harvest groups from T3 and T4. No other noteworthy differences in osmolality were evident.

Haemoglobin ranged from 12.2 ± 1.3 to 22.6 ± 1.2 g/dL. Only one minor difference was evident with the levels in harvest fish of T5 being significantly lower than at the end tow of T1.

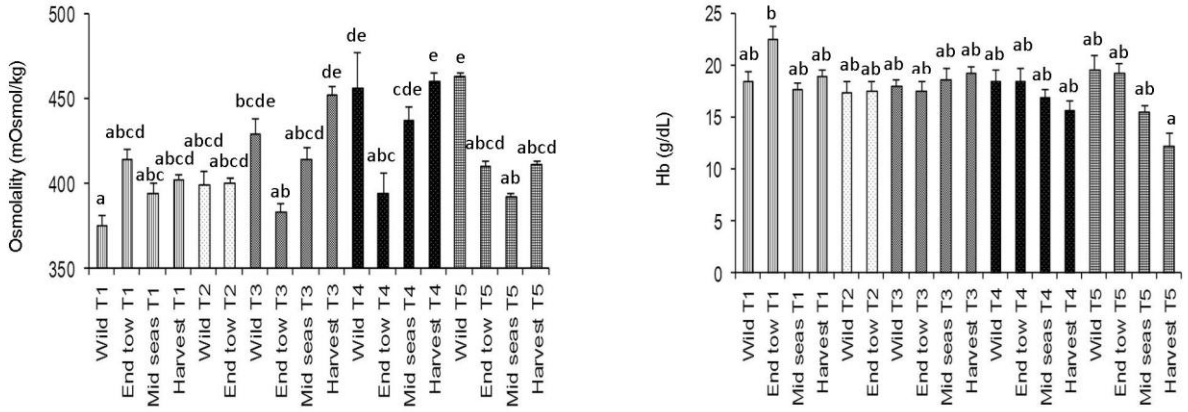


Figure 3.9 2007 osmolality and Hb levels in SBT from various tows (T1, T2, T3, T4, T5) sampled at seasonal ranching stages (wild, end tow, mid seas[on], harvest). Error bars show standard error of mean. Labels group means that do not differ *p=0.01*

IGF levels for groups sampled in 2007 are shown in Figure 3.10. Levels ranged from 24.7 ± 1.6 to 45.0 ± 1.6 ng/mL. Unfortunately samples were not available for 3 of the wild fish groups (T3, T4 and T5). The highest IGF levels were recorded in groups from the later stages of ranching, with those from harvest fish of T3 and mid season fish of T4 being significantly higher than those of seven of the other groups. The lowest IGF level recorded was in the end tow group of T2 which was significantly lower than the levels in the groups of wild T1, mid season T1, mid season T3, harvest T3, mid season T4, harvest T4 and mid season T5. Three of the four lowest IGF levels came from end tow groups (end tows T1, T2 andT5).

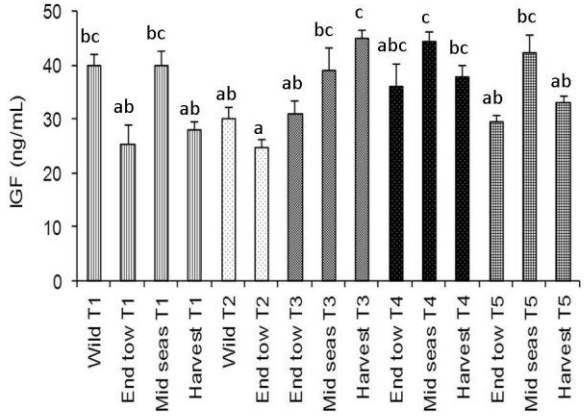


Figure 3.10 2007 IGF levels in SBT from various tows (T1, T2, T3, T4, T5) sampled at seasonal ranching stages (wild, end tow, mid seas[on], harvest). Error bars show standard error of mean. Labels group means that do not differ *p=0.01*

3.3.3 2008 indicators

Cortisol and glucose levels in groups sampled in 2008 are shown in Figure 3.11. Cortisol ranged from 8.1 $\pm$ 2.5 to 148.6 $\pm$ 39.9 ng/mL. There was considerable variation in cortisol levels amongst similar ranching stages across the four tows however harvest levels are significantly higher than mid season levels in all tows except for tow 4. End tow levels in T1 and T5 were similar to these harvest groups. Glucose levels ranged from 5.5 $\pm$ 0.1 to 9.5 $\pm$ 0.8 mmol/L and were significantly higher in the end tow groups than at harvest in three of the four tows (T2, T4 and T5).

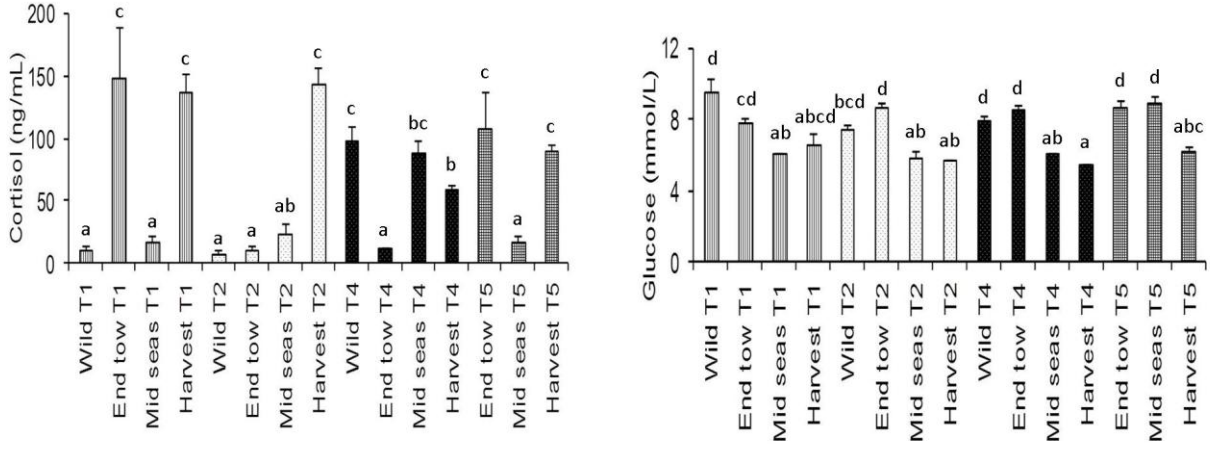


Figure 3.11 2008 cortisol and glucose levels in SBT from various tows (T1, T2, T4, T5) sampled at seasonal ranching stages (wild, end tow, mid seas[on], harvest). Error bars show standard error of mean. Labels group means that do not differ *p=0.01*

Lactate and pH levels for 2008 are shown in Figure 3.12. Lactate levels ranged from 1.2 $\pm$ 0.2 to 12.9 $\pm$ 1.0 mmol/L and were significantly higher at harvest in all of the tows than any of the early ranching stages, excluding wild fish from T1.

The pH levels ranged from 7.4 ± 0.1 to 8.8 ± 0.04 and in three of the four tows sampled in 2008 (T1, T2 and T4), pH levels were significantly higher at end tow than subsequent sampling mid season and at harvest.

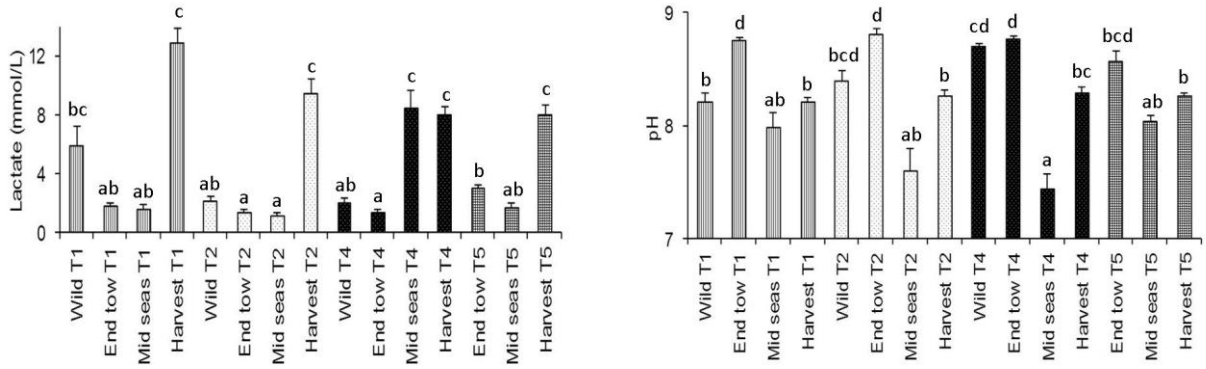


Figure 3.12 2008 lactate and pH levels in SBT from various tows (T1, T2, T4, T5) sampled at seasonal ranching stages (wild, end tow, mid seas[on], harvest). Error bars show standard error of mean. Labels group means that do not differ *p=0.01*

Lysozyme and IGF levels in groups sampled in 2008 are shown in Figure 3.13. Note in the display of results for previous cohorts IgM has been paired with lysozyme. As no IgM results were available for 2008 (Table 3.4) the results for IGF are presented below for symmetry.

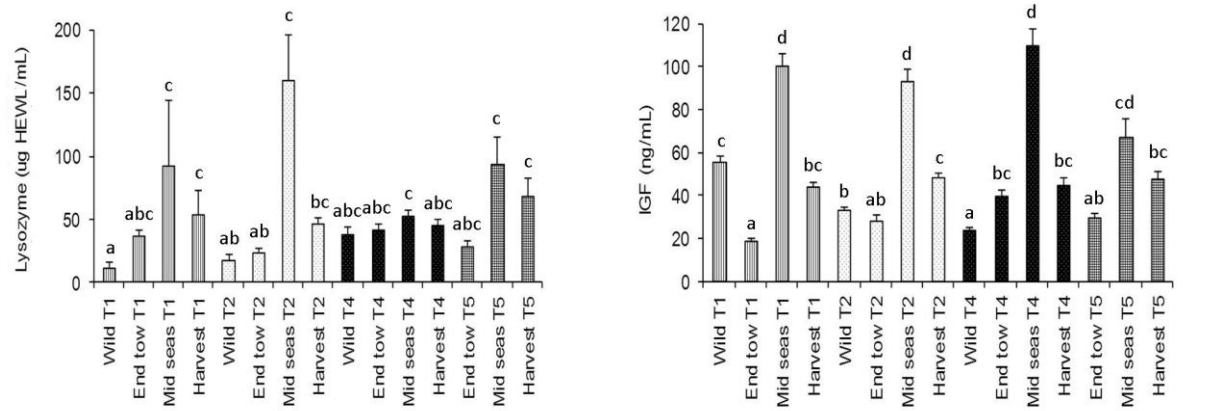


Figure 3.13 2008 lysozyme and IGF levels in SBT from various tows (T1, T2, T4, T5) sampled at seasonal ranching stages (wild, end tow, mid seas[on], harvest). Error bars show standard error of mean. Labels group means that do not differ *p=0.01*

Lysozyme ranged from 11.5 ± 4.7 to 160.5 ± 36.0 µg HEWL/mL and in two of the four tows sampled (T1 and T2) mid season lysozyme levels were significantly higher than wild fish. IGF levels ranged from 24.2 ± 1.2 to 109.9 ± 8.2 ng/mL and again mid season IGF levels were significantly higher than at early ranching stages for all of the four tows sampled and significantly higher than at harvest for T1, T2, and T4.

Osmolality and haemoglobin levels for groups sampled in 2008 are shown in Figure 3.14. Osmolality ranged from 405.2 ± 3.8 to 484.2 ± 11.0 mOsmol/kg. In two of the tows sampled (T1 and T5) osmolality was significantly higher at harvest than at end tow or mid season.

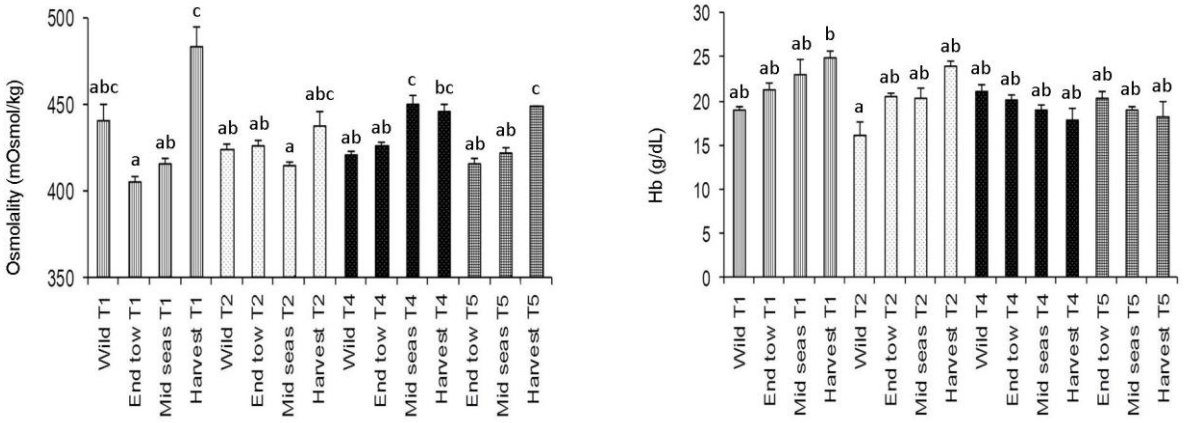


Figure 3.14 2008 osmolality and Hb levels in SBT from various tows (T1, T2, T4, T5) sampled at seasonal ranching stages (wild, end tow, mid seas[on], harvest). Error bars show standard error of mean. Labels group means that do not differ *p=0.01*

Tow 4 had significantly higher osmolality at mid season than at earlier ranching stages. Haemoglobin ranged from 16.3 ± 1.4 to 24.9 ± 0.8 g/dL and with the minor exception of the harvest fish of T1 having significantly higher levels than the wild fish of T2, no difference was evident in the haemoglobin levels of other groups sampled in 2008.

3.3.4 Differences in indicators of different ranching stages

To investigate the differences in stress and immune response indicators of different ranching stages the data on response indicators from each company for each of the ranching stages sampled for the three cohorts were pooled and reanalysed as described in Section 3.2. This approach also was believed to be more informative than simply pooling all stages from all companies to test for any differences between the three cohorts of 2006, 2007 and 2008.

Mean cortisol and glucose levels of SBT from the various pooled ranching stage groups for each of the three cohorts is provided in Figure 3.15. Cortisol ranged from 13.7 ± 3.1 to 231.4 ± 40.2 ng/mL. End tow and harvest cortisol levels in 2007 were significantly higher than the three pre-harvest groups in 2008 (wild, end tow and midseason) and also the wild and harvest SBT of 2006.

All of the pooled 2007 cortisol levels were significantly higher than those of the wild groups of 2006 and 2008 and the mid season groups of 2008. Glucose ranged from 5.0 ± 0.1 to 8.5 ± 0.1 mmol/L and the levels of the SBT from the post-wild groups of 2007 (end tow, mid season and harvest) were significantly lower than the pre-harvest groups in 2008 (wild, end tow and mid season) and the post-wild groups in 2006. The wild and end tow groups of 2008 had significantly higher glucose levels than all other cohort groups with the exception of end tow 2006 and mid season 2008.

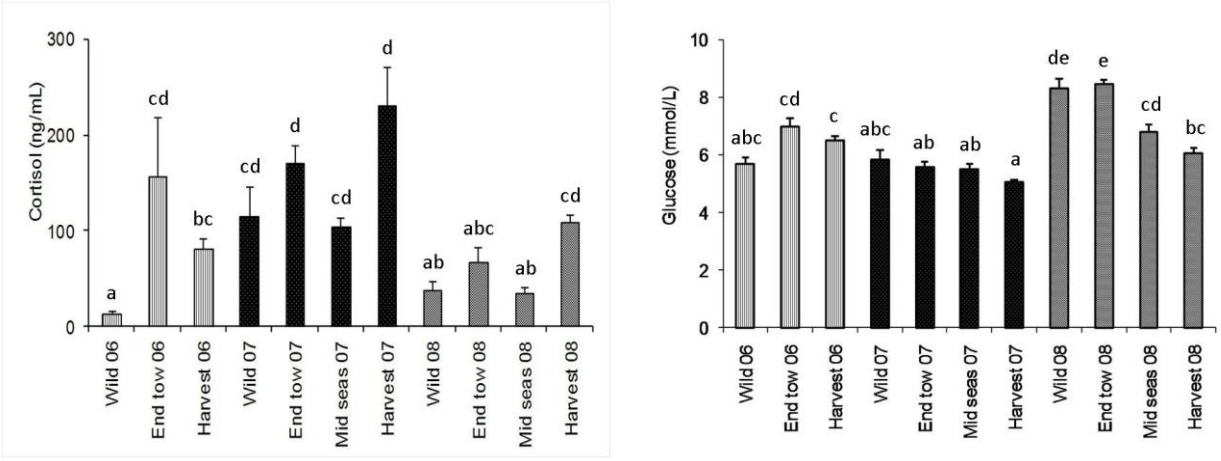


Figure 3.15 Cortisol and glucose levels in SBT from 2006 to 2008 sampled at seasonal ranching stages (wild, end tow, mid seas[on], harvest). Pooled data from all tows sampled. Error bars show standard error of mean. Labels group means that do not differ *p=0.01*

Mean lactate and pH levels of SBT from the various ranching stage groups (pooled within years) are presented in Figure 3.16. Lactate ranged from 1.9 ± 0.1 to 11.4 ± 1.0 mmol/L Harvest groups in 2006 and 2008 had significantly higher lactate levels than any other pooled groups in the three cohorts. End tow lactate levels were significantly lower than any of the three cohort's harvest group levels.

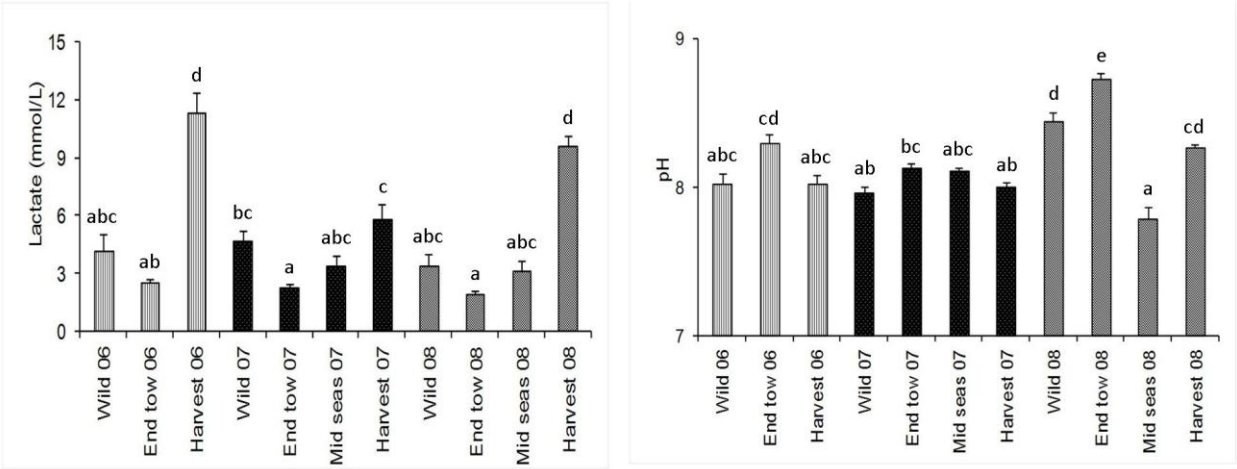


Figure 3.16 Lactate and pH levels in SBT from 2006 to 2008 sampled at seasonal ranching stages (wild, end tow, mid seas[on], harvest). Pooled data from all tows sampled. Error bars show standard error of mean. Labels group means that do not differ *p=0.01*

Mean pH levels ranged from 7.8 ± 0.1 to 8.7 ± 0.03 and the end tow groups in 2008 were significantly higher than any of the other groups sampled in the three cohorts. No other definitive result was evident in the pooled pH analyses.

Mean lysozyme and IgM levels of SBT from the various ranching stage groups (pooled within years) are presented in Figure 3.17. Lysozyme levels ranged from 7.8 ± 1.2 to 101.6 ± 17.4 µg HEWL/mL. The mid season groups of 2007 and 2008 were significantly higher than all other groups (excluding the harvest 2008 groups) sampled in the three cohorts. Note mid season groups were not sampled in the first cohort of the study in 2006. Wild SBT lysozyme levels in each of the three cohorts were significantly lower than any of the mid season or harvest groups sampled over the three cohorts. IgM ranged from 3.5 ± 0.8 to 23.2 ± 10.2 mg/mL and there was no significant difference in the pooled IgM levels of the ranching stages for the two cohorts studied.

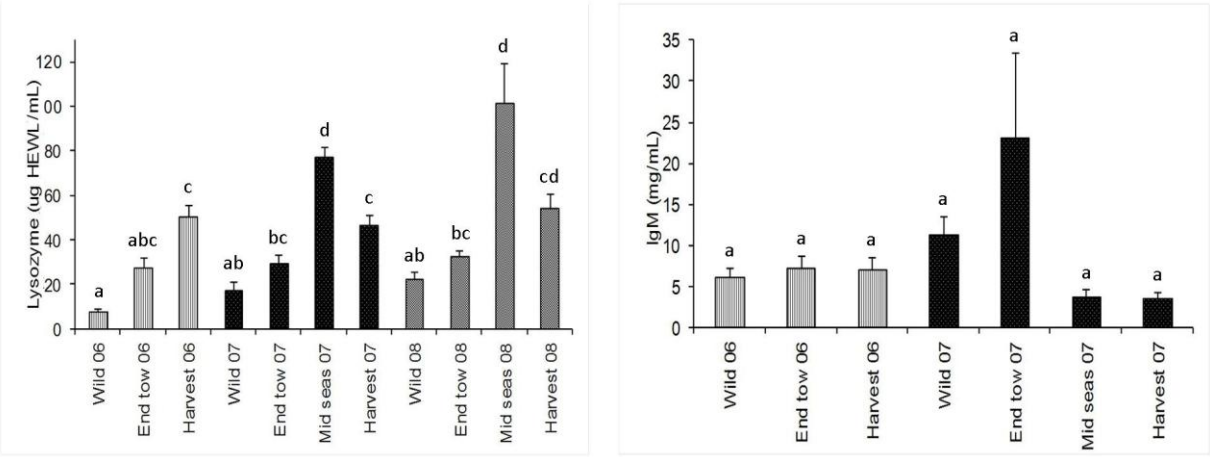


Figure 3.17 Lysozyme and IgM levels in SBT from 2006 to 2008 sampled at seasonal ranching stages (wild, end tow, mid seas[on], harvest). Pooled data from all tows sampled. Error bars show standard error of mean. Labels group means that do not differ *p=0.01*

Mean blood osmolality and Hb of SBT from the various ranching stage groups (pooled within years) are presented in Figure 3.18. Osmolality ranged from 375.0 ± 6.6 to 454.5 ± 4.7 mOsmol/kg and were significantly higher in harvests groups in each year and all the 2008 pooled groups than the early stages sampled in 2006 (wild and end tow).

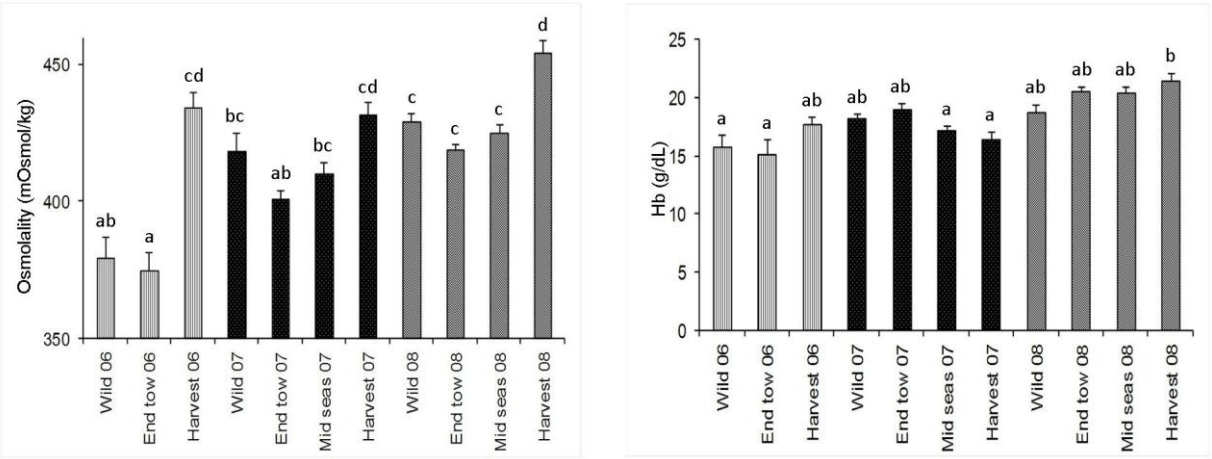


Figure 3.18 Osmolality and Hb levels in SBT from 2006 to 2008 sampled at seasonal ranching stages (wild, end tow, mid seas[on], harvest). Pooled data from all tows sampled. Error bars show standard error of mean. Labels group means that do not differ *p=0.01*

Haemoglobin levels ranged from 15.2 $\pm$ 1.3 to 21.4 $\pm$ 0.8 g/dL and there was no difference in Hb levels across the sampled groups with the exception of the harvest groups in 2008 being significantly higher than the wild and end tow groups of 2006 and the mid season and harvest groups of 2007.

Mean IGF levels of SBT from the various ranching stage groups (pooled within years) are presented in Figure 3.19. IGF levels ranged from 15.2 $\pm$ 1.9 to 92.2 $\pm$ 4.4 ng/mL and in 2006 were significantly lower than either of the subsequent two cohorts (2007 and 2008). The mid season group of 2008 had significantly higher levels than any other group.

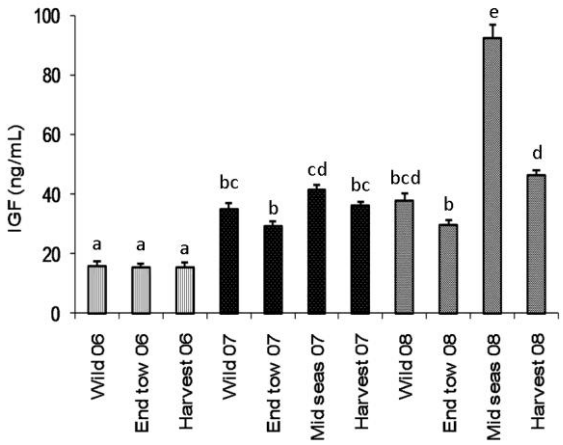


Figure 3.19 IGF levels in SBT from 2006 to 2008 sampled at seasonal ranching stages (wild, end tow, mid seas[on], harvest). Pooled data from all tows sampled. Error bars show standard error of mean. Labels group means that do not differ *p=0.01*

The relationship between individual stress and immune response indicators of SBT over the study period was of interest. Table 3.5 provides a correlation matrix of Spearman's r_s coefficients for the indicators. A significance level of $\alpha = 0.01$ again was adopted and significant associations are highlighted in red.

Table 3.5 Correlation matrix for stress and immune response indicators for 2006 to 2008.
Top value in each cell is Spearman's rho coefficient, bottom value in each cell is the 2-tailed level of probability. Significant correlations are shown in red. *p=0.01*. Cort = cortisol, Gluc = glucose, Lac = lactate, Lys = lysozyme, IgM = Immunoglobulin M, Osmo = osmolality, Hb = haemoglobin, IGF = Insulin-like growth factor.

Cortisol	Cort							
Glucose	*-0.163* *0.001*	Gluc						
Lactate	*0.335* *<0.001*	0.079 0.117	Lac					
pH	-0.082 0.102	*0.268* *<0.001*	*-0.207* *<0.001*	pH				
Lysozyme	*0.136* *0.007*	-0.014 0.788	-0.013 0.804	-0.053 0.296	Lys			
IgM	-0.144 0.024	0.026 0.686	-0.082 0.200	-0.004 0.956	*-0.300* *<0.001*	IgM		
Osmolality	*-0.141* *0.005*	*0.215* *<0.001*	*0.562* *<0.001*	-0.059 0.244	*0.145* *0.004*	-0.038 0.555	Osmo	
Hb	0.200 0.689	*0.166* *0.001*	0.054 0.285	0.114 0.025	0.015 0.774	0.062 0.337	*0.168* *0.001*	Hb
IGF	-0.027 0.612	-0.069 0.188	0.026 0.613	*-0.174* *0.001*	*0.327* *<0.001*	*-0.220* *0.001*	*0.333* *<0.001*	*0.161* *0.002*

Most of the correlations were very weak with a Spearman's r_s coefficient of ≤ 0.19. These included very weak positive correlations between cortisol and lysozyme, between osmolality and both lysozyme and Hb, and between Hb and both glucose and IgF. There were very weak negative correlations between cortisol and both glucose and osmolality, and between pH and IGF.

There were weak positive correlations ($0.20 \leq r_s \leq 0.39$) between cortisol and lactate, between glucose and both pH and osmolality and between IGF and both lysozyme and osmolality. Weak negative correlations were between pH and lactate and between IgM and both lysozyme and IGF. The strongest association with a $r_s = 0.562$ was the moderate positive correlation between lactate and osmolality.

3.4 DISCUSSION

The importance of measuring base levels of stress and immune response indicators in wild fish has been emphasised. With possibly the exception of cortisol and IgM, the base levels measured in wild SBT during this study compared favourably with those reported previously for other bluefin tunas. Cortisol levels in wild and captive ABT were reported as part of the REPRO-DOTT breeding program in the Mediterranean (Anon 2007). Levels varied greatly and were very high in wild fish (6 to 600 ng/mL) presumably as a result of handling and sampling artefacts (Section 3.1). The REPRO-DOTT Final Report considered a value of 50 ng/mL to be representative of a pre-stress cortisol level in ABT.

Cortisol levels in wild SBT of the three cohorts sampled in this study ranged from 8.4 $\pm$ 3.6 to 264.4 $\pm$ 146.9 ng/mL (Table 3.4). Despite the best efforts to sample fish within a couple of minutes of hooking on the fishing grounds, it is likely the highest cortisol levels recorded from the groups wild T2 in 2007 (135.3 $\pm$ 38.3 ng/mL), wild T4 in 2007 (264.4 $\pm$ 146.9 ng/mL) and wild T4 in 2008 (97.9 $\pm$ 12.2 ng/mL) are representative of the onset of an acute handling primary stress response. The unknown latency period for the onset of a stress response in SBT complicates interpreting such results. Response times to detect statistically significant elevations of cortisol in some fish can be as short as 2.5 minutes and response latencies appear to be related to metabolic/trophic levels (Pankhurst 2011), with sedentary bottom dwellers having longer latency periods. The time series sampling of cortisol levels to determine the latency of the response has not been carried out for SBT (and conceptually would appear difficult), however the latency conceivably could be shorter than many other fish given the higher metabolic rates and endothermic capability of bluefin tunas (Section 1.1). Notwithstanding the above caveat, a suggested pre-stress reference range for wild SBT cortisol levels from this study is 10 to 70 ng/mL.

Total immunoglobulin (Ig) levels in wild SBT in the mid 1990s were reported as 0.7 to 1.3 mg/mL (Watts et al 2002; approximate values taken from graphical representations) whereas IgM levels in wild SBT in this study were higher, ranging from 3.7 $\pm$ 2.2 to 16.4 $\pm$ 8.6 mg/mL. The reason for this apparent shift in the base level of immunoglobulin in wild IgM from the mid 1990s to the mid/late 2000s is unknown. However low level stress and/or the onset of an antigenic challenge can lead to enhanced innate immune function (Nardocci et al 2014) and it is possible that an environmental change over this time in the GAB has initiated such an increase in immunocompetence. Observed recent environmental and anthropogenic changes in

the GAB are discussed in Chapter 6 within this context. A suggested reference range for IgM for wild SBT from this study is 4 to 16 mg/mL. The remaining stress and immune response indicators measured in wild SBT in this study had levels very similar to those previously published for ABT and SBT (Table 3.1). Suggested reference ranges are for glucose 5 to 10 mmol/L, for lactate 1 to 10 mmol/L, for pH 7.5 to 8.5, for lysozyme 5 to 40 µg HEWL/mL, for haemoglobin 13 to 21 g/dL, for osmolality 375 to 460 mOsmol/kg and for IGF 12 to 55 ng/mL.

Previous work has reported significant differences in some of these 'health' indicators in wild SBT between cohorts from different years and even from between different schools of the same year (Kirchhoff 2014). That study indicated that eighty four percent of the variation in SBT health when considering all measured variables could be explained by grouping fish by the year of study, and in one year (2012) schools of wild SBT had significantly different Hb, pH, osmolality and lactate levels. In considering the pooled data analysis (Section 3.2.4) a number of significant differences between years in measured variables of wild SBT were evident; cortisol in 2007 wild SBT was significantly higher than in 2006 or 2008 wild SBT, glucose in 2008 wild SBT was significantly higher than in 2006 or 2007 wild SBT, pH in 2008 wild SBT was significantly higher than in 2006 or 2007 wild SBT; osmolality in 2008 wild SBT was significantly higher than in 2006 wild SBT (2007 wild SBT with intermediate levels) and IGF was significantly higher in 2007 and 2008 wild SBT, confirming at least modest differences in the 'health' of yearly cohorts of wild SBT captured for farming. A general trend is that a majority of stress response indicators were higher in wild SBT sampled in 2008 than previous years. Possible commercial implications from this observation on the different 'health' status of wild SBT are considered in Chapter 6.

In captive fish cortisol frequently has been measured in stress studies because of its central position in the primary neuroendocrine response to challenge and the myriad of influences it has on physiological functions including regulation of carbohydrate, protein and amino acid metabolism and on osmoregulation through ion exchange processes in the gills (Ellis et al 2012, McCormick 2001). It also influences immune responses such as the expression and activity of pro and anti-inflammatory cytokines; phagocytosis, leucocyte activity and IgM secretion by lymphocytes (Nardocci et al 2014).

Pankhurst (2011) emphasised that a broad range of response in cortisol levels can be elicited by stressors and this was indeed the case in this study. Wide variation in cortisol levels were found both between ranching groups of each cohort and even within groups of SBT at the same ranching stage. Some of the variation, particularly in wild SBT, has been attributed to sampling artefact and more information is required on the latency of the cortisol response to assess the extent of sampling influence on levels measured under ranching conditions.

Notwithstanding this, in the two cohorts (2007 and 2008) with sampling at all four of the identified ranching stages (wild, end tow, mid season and harvest), mid season SBT cortisol levels generally were significantly lower than harvest SBT levels and, in approximately half of the tows sampled, end tow cortisol levels were significantly higher than mid season. There appears to be an acute primary cortisol response as a result of the tow conditions followed by some degree of habituation (reduction) in the cortisol response. Such habituation has been reported previously for the cortisol response during chronic stress. Repeated handling stress over an 8 week period indicated a strong habituation in the cortisol response of Eurasian perch *Perca fluviatilis,* and rainbow trout *Oncorhynchus mykiss*, (see Jentoft et al 2005) and of carp *Carassius auratus gibelio,* over 5 months (Yang et al 2015). A similar habituation in the cortisol response of Atlantic salmon *Salmo salar*, has also been reported during chronic hypoxia stress (Kvamme et al 2013).

Despite the suggested habituation, cortisol levels remain modestly elevated above the suggested pre-stress reference range (35.6 $\pm$ 5.8 to 104.1 $\pm$ 9.2 ng/mL compared to a reference range of 10 to 70 ng/mL) through-out the ranching period and it appears a low level, chronic primary cortisol response endures. The peak in levels again at harvest likely are a result of the higher sustained activity forced upon the SBT during the crowding in the harvest net and capture by diver and represent another acute stress response. The acute response during tows is likely from higher stocking densities in the tow cage (see Table 1.4 for examples). Another possibility for the cortisol response measured at end tow is an increased cortisol level as a result of fasting (Barcellos et al 2010) if feeding is inadequate on tows. Differences in the access of individual SBT to feed on tow, and low average feed intake could be expected from a restricted feed supply. It is suggested such nutritional deficits could have played a part in both the high variation of cortisol levels measured within groups (in 2006) and the elevated average cortisol levels reported for the end tow SBT groups. Unfortunately company confidentiality prevented feeding levels on tows being an identified tow variable in this study.

Only two of the previous studies on SBT stress have reported cortisol levels. Thomas et al reported very low levels (4.74 $\pm$ 2.18 ng/mL) 6 weeks after transfer in ranched SBT in the late 1990s. These levels suggest the SBT sampled were not displaying a stress response. Kirchhoff et al (2011b) in a study of ranched SBT in 2009 reported wide variation in cortisol levels with no statistically significant difference between end tow groups and an early mid season sampling point (8 weeks) and significantly higher cortisol levels at harvest. Despite the statistical caveat, all 3 of the end tow groups in each of the tow cohorts sampled in that study had higher cortisol levels than the corresponding mid season groups suggesting some degree of habituation. Mid season cortisol levels ranged from approximately 30 to 115 ng/mL in one tow and 30 to 60 ng/mL in the other (Kirchhoff 2011b, values transposed from graphical representations) indicating a low level chronic primary stress response endured in only the former SBT group.

Unlike cortisol, the secondary response glucose levels in SBT were affected only modestly by ranching conditions in this study. At all times glucose levels were within acceptable glycaemia limits for fish (Polakof et al 2012), were within the reference range suggested for pre-stressed wild SBT (5 to 10 mmol/L) and in two of the three cohorts sampled (2006 and 2007) there was no change in glucose levels of any SBT group throughout the ranching year from capture to harvest. In three of the four tows in 2008 however a secondary stress response of increased glycaemia was evident at end tow. It is suggested again that this may be related to differences in the health status of the wild SBT cohorts of 2008 as the tow analysis in Chapter 2 found no obvious differences between the characteristics of tows in the latter years of the study (2007 and 2008) and those of earlier years (2003 to 2006).

At harvest there was no elevated glycaemia suggesting the timing differential between the primary cortisol response measured and the onset of a secondary response rise in glucose levels was greater than the period required to confine and euthanise fish. Cage cultured bronze gudgeon (*Coreius guichenoti*) exhibited an immediate elevation in cortisol level and a peak within one hour following handling stress, and a three hour delay in the secondary elevation in glycaemia (Zhao et al). Quicker response times for glucose increases of within 15 minutes of handling in coral trout *Plectropomus leopardus* (see Frisch and Anderson 2000) and within 30 minutes in Eurasian perch *Perca fluviatilis*, and within 60 minutes in rainbow trout *Oncorhynchus mykiss*, (see Jentoft et al 2005) have been reported.

Studies on SBT cohorts from years subsequent to this study (after 2008) have reported elevated glucose levels during ranching stages compared to wild fish (Kirchhoff et al 2011a, Kirchhoff et al 2011b, up to 30% higher, Kirchhoff et al 2011c). It is suggested that the difference in result for the glucose stress response for various SBT ranching cohorts from 2006 to 2008 (this study) and for 2009 and 2010 reflected either a difference in the health status of wild fish as described above, or a change in the stress profile for SBT under ranching conditions. This is considered further in Chapter 6.

Plasma lactate levels were higher in harvest groups than other ranching stages throughout most of the tows sampled during the three cohorts. In the pooled analysis lactate levels were significantly higher at harvest than the end tow stages. Kirchhoff et al (2011c) reported a similar finding for lactate levels in SBT, finding no significant difference in levels in the first 8 weeks of ranching but significantly higher levels at harvest. It is possible the higher lactate level in harvest groups resulted from the forced activity following crowding and the eventual build up in lactate from anaerobic glycolysis. However there was an increasing trend in lactate from end tow and mid season through to harvest and it is likely the higher levels at harvest represent a chronic stress that ensues following the release and utilisation of stored energy in the initial months of ranching. Blood pH levels of sampled SBT groups varied within a narrow range throughout the study. No significant differences were evident between groups in 2006 and 2007 with the exception of one wild group in 2007 (T5) that had lower pH and one end tow group with significantly higher (T4). Kirchhoff et al (2011c) reported pH in captive SBT were elevated at 7 weeks but considered the level of 7.99 $\pm$ 0.03 (cf 8.45 $\pm$ 0.03 end tow T4 in this study) to be of no physiological significance. This study supported the conclusion that pH changes measured under ranching conditions were of no physiological significance.

Possibly the most noteworthy change in SBT health over the course of the study was in one measure of immune system activity, the anti-microbial enzyme lysozyme. This lytic enzyme is an important defence against bacterial antigens, is an opsonin activating the complement system and phagocytes and is present in mucus, lymphoid tissue, plasma and other body fluids of most fish species (Magnadóttir 2006, Whyte 2007, Saurabn and Sahoo 2008). Lysozyme levels routinely were elevated in the mid season and harvest groups. A similar elevation in lysozyme levels as the grow-out period continues has been reported in several other studies. Watts et al (2002) reported an increase in lysozyme as length of time in captivity increased (20 to 28 µg/mL in wild fish to up to 41 to 54 µg/mL). Hayward et al (2010) reported elevated lysozyme levels in association with increasing infection intensity of the blood fluke *Cardicola*

forsteri. Kirchhoff et al (2012) found lysozyme levels rose from 9.4 $\pm$ 2.1 µg/mL in wild fish to as high as 296.0 $\pm$ 63.7 µg/mL only 5 weeks into grow-out. Another study reported the same trend with lysozyme levels rising from approximately 25 µg/mL at end tow to 220 µg/mL at 8 weeks (Kirchhoff et al 2011b; values transposed from graphical representations) and it has been suggested that the rise in lysozyme (and ACH50) activity in the initial months after transfer were in response to egg production of *C. forsteri*, estimated to be at 30 days post infection (Kirchhoff et al 2011a). The sampling peak in lysozyme level in this study was considerably later than that of those mentioned above from latter years. Kirchhoff et al (2011c) reported that following the peak in lysozyme at 5 weeks, levels decreased coinciding with a peak in mortalities of SBT and suggested this represented either the utilisation of such defence molecules in meeting the challenge of infection and/or a lessening in the molecule production ability of the host. The increase in lysozyme levels with time in grow-out in this study suggested a similar increased exposure to antigens or an infectious agent.

Immunoglobulin was the other immune response variable measured over the course of this study. Anecdotal trends from 2007 suggested higher IgM levels in the wild and end tow groups than later in the ranching cycle, however no statistically significant response to ranching conditions was evident in the first two cohort years 2006 and 2007 and this indicator was dropped from the study prior to the 2008 sampling. Minor differences reported in haemoglobin levels over the course of the study were not considered to be related to stress or ranching practices. Hb levels were within the range reported previously for SBT of 13 to 18g/dL (Rough et al 2005) and 22 to 28 g/dL (Kirchhoff et al 2014).

Osmolality was significantly higher in harvest groups. Elevated cortisol levels influence the ion exchange capabilities of the gills (McCormick 2001) although the very weak negative correlation between cortisol and osmolality in this study suggests a more indirect influence. It is likely a combination of secondary stress responses (increasing lactate, increasing lysozyme) contribute to increasing the osmoregulatory burden. Other studies have reported similar elevations in osmolality at harvest (Kirchhoff 2011a; 20 mOsmol/kg increase) or no differences in osmolality of SBT under ranching conditions (Kirchhoff 2011b, 2011c).

Reductions in performance measures such as growth represent a tertiary stress response and are of obvious interest to fish farmers. Insulin-like growth factor (IGF) levels peaked mid season in the ranching cycle, and are lowest at end tow. Circulating IGF1 levels in fish have been shown to change with varying nutritional status and feed consumption, with fasting

decreasing levels (Beckman 2011, Kawaguchi et al 2013). This may explain the lower, end tow levels as optimal feed practices are not feasible on tow. As a measure of growth capacity well into the ranching cycle, the high mid season IGF levels suggest that the growth potential of SBT under ranching conditions had not been compromised markedly by the series of stressors to which they had been subjected to since capture.

Most of the correlations between the stress and immune response indicators were modest at best. This partially may be ascribed to the inherent lability of the stress responses within individuals of a species (Pankhurst 2011) which can be compounded by interactions (social, physiological and physical) within an aquaculture environment. The strongest association appeared to be between osmolality and a number of other indicators (possibly singularly or in combination) including glucose, lactate, lysozyme, Hb and IGF. The next level of association was between cortisol and lactate and the axis, increasing cortisol → increasing lactate → impaired osmoregulation was the most evident in SBT under ranching conditions in this study.

However SBT appear to cope with the increased burden on osmoregulation in terms of production capacity as there is no evidence of a drop in grow potential, as IGF increased with osmolality (r_s = 0.333). As lactate levels increase there is a concomitant (albeit weakly associated) drop in pH (r_s = -0.207), presumably as a result of mild acidosis from increased lactic acid in the blood stream. One instance of suggested acidosis in SBT at harvest was reported by Kirchhoff et al (2011b). An interesting observation was the comparatively small part a glucose response had in the stress response of SBT under ranching conditions in this study. This is considered further in Chapter 6.

The towing of SBT after capture presents a measureable stress on the captive fish as evident from the elevated cortisol levels at the end tow point. The continuing elevated cortisol (albeit with some habituation) through mid season, and elevated lactate at harvest suggests a continuing chronic stress from ranching conditions that the SBT's immune system is addressing as evident with the measured increase in lysozyme activity. An additional burden is placed upon the osmoregulatory capacity although the peak in IGF through mid season indicates that the SBT are at least coping with the challenges presented through ranching grow-out. The obvious extension to the above query was the influence that holding SBT for a longer period of time than the normal 6 to 8 months would have on SBT health as measured by these stress and immune response indicators. This is considered in Chapter 4.

CHAPTER 4 LONG - TERM HOLDING OF SBT

4.1 INTRODUCTION

By world definitions SBT ranching is a seasonal fattening cycle, catching SBT in December to March and completing harvest by September, a maximum of 10 months in captivity. The majority of SBT are held for only 6 to 8 months before harvest. Mediterranean ABT operations are distinguished as the fattening of large fish (> 80 kg) for 5 to 8 months, or the farming of smaller fish in Croatia (> 8 kg) for up to several years (Mylonas et al 2010). The sashimi market places a price premium on larger fish (ASBTIA pers comm), explaining the preparedness of Croatian operators to endure extended periods of meeting the fish farmer's challenge to maximise size and production.

As all of the world's bluefin tuna capture-based aquaculture sectors have restricted catch quotas (ICCAT 2015, IATTC 2014, CCSBT 2013), the only two options for substantive increases in production are by stocking economically viable hatchery seed stock or increasing the individual size of the output product from grow-out. Section 2.1.1 summarised the difficulties in achieving the former, and the figure of 97.4 % of Japanese PBT aquaculture production in 2014 coming from wild sourced seed stock is a stark reminder of the work ahead in economic hatchery production of bluefin tuna (Table 2.2). The only obvious alternative available to the SBT ranching sector has been to investigate the economic, operational and biological feasibility of holding SBT over an extended period of time so that a larger product at harvest was available.

SBT tuna ranching production from 2000 to 2014 is shown in Figure 4.1 (ASBTIA pers comm). The quota restricted input into SBT grow-out operations is shown by the white columns. Note a decline in TAC quota is behind the reduction in input from 2009 to 2013 rather than any operational decisions. The whole weight production in tonnes from SBT ranching each year is shown by the grey columns. It is obvious that there has only been a modest and inconsistent increase in production by SBT ranching operations since at least 2000.

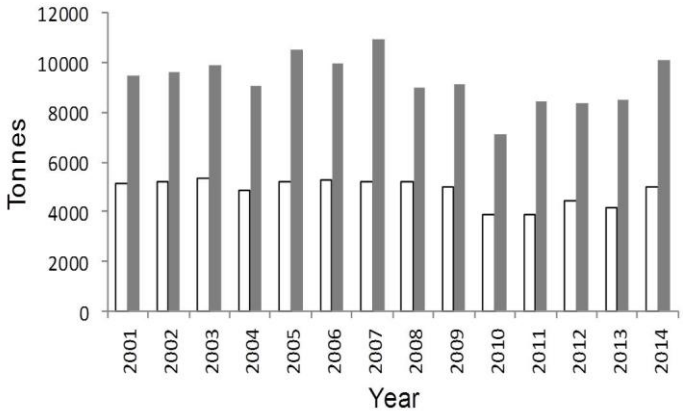


Figure 4.1 Australian tuna ranching production (tonnes) since 2000. White columns show tonnes of SBT into grow-out operations. Grey columns show tonnes of SBT (whole weight) out of grow-out operations. Source ASBTIA pers comm

The extended grow-out period model of farming ABT in Croatia has been denoted long-term holding (LTH) in the SBT industry. The feasibility of LTH was investigated in an Aquafin CRC/FRDC sponsored project from February 2005 to August 2006. The study looked at diet, growth, parasite interactions and health aspects of holding SBT for an extended period, particularly over the warmer summer months (Ellis et al 2009b, Hayward et al 2008a, Hayward et al 2009). Of interest for this study were the stress and immune responses of SBT under LTH.

4.2 METHODS

4.2.1 Study design

SBT for the LTH trial were caught on the fishing grounds in February 2005. To accommodate the various research areas of interest the 2005 cohort was towed to Port Lincoln and transferred by hook capture into four research sea cages. These original four groups were maintained for the first 9 months as part of a dietary manipulation study.

At the cessation of the dietary study (October) all tuna remaining in the four research cages were transferred into a single combined cage of 32 m diameter, where they were maintained until August 2006. Logistics, and the need to gain as much information as possible in several research disciplines from the limited number of research SBT available, mandated this unconventional experimental design of combining the groups midway through the grow-out period. Opportunistic sampling of the four research cages and then the subsequent combined cage were undertaken as summarised in Table 4.1. Approximate densities were 0.023 fish/m^3 in Cages 1 to 4 and 0.062 fish/m^3 in the combined cage. Sample sizes are shown.

Table 4.1 Summary of LTH trial showing sampling times and sampling sizes. Month is approximate time after capture with end tow being one month

Month	2005 cohort					
	End tow	Cage 1	Cage 2	Cage 3	Cage 4	Combined
1	10					
3		10	10	10	10	
4.5		10	10	10	10	
6		10	10	10	10	
9						30
13						10
18						30

4.2.2 Analytical methods

Stress and immune response indicators described in earlier chapters were measured including cortisol, glucose, lactate, pH, lysozyme, IgM, osmolality, Hb and IGF. Blood sampling and laboratory analytical techniques were as described in Sections 3.2.1 and 3.2.2 respectively. Because of the extended holding time, additional immune response indicators, complement activity (CH50) and alternative complement activity (ACH50) were added at the start of the trial although problems with laboratory procedures meant analytical results were only obtained for the first six months.

Measurement of complement activity followed that of Watts et al (2002). Briefly, sheep red blood cells (SRBC) were isolated, washed in sodium phosphate dibasic (Na_2PO_4) and a salt (NaCl) solution and resuspended in a suitable buffer. For the complement haemolysis (CH) measurement a Hanks balanced salt solution (HBSS) was used and for the alternative complement haemolysis (ACH) measure a CH inhibitor was added to HBSS. Cell suspensions were incubated for 1 hour at 25 ^{o}C in wells of a microtiter plate, centrifuged to stop the reaction and supernatant transferred to wells of a Titertek Multiscan MC plate reader and absorbance measured at 450 nm. One unit of activity was defined as the amount of undiluted serum resulting in 50% lysis of a standard SRBC suspension and the number of CH50 and ACH50 units/mL determined from a plot of serum dilutions against mean haemolysis for each serum.

4.2.3 Statistical inference

An initial series of 2 way factorial ANOVA analyses were undertaken to test the appropriateness of combining the 4 groups at the end of the dietary studies and of pooling data from SBT of the dietary trial cages for subsequent analyses. Independent factors were Cage (4 levels - Cages 1, 2, 3 and 4) and Months held in ranching (3 levels - 3, 4.5 and 6 months) and individual indicators as the dependent variables. A conservative decision making probability of $\alpha = 0.01$ significance again was used. Probability levels for F-values of each indicator were cortisol $p = 0.54$, glucose $p = 0.12$, lactate $p = 0.11$, pH = 0.15, lysozyme $p \leq 0.01$, osmolality $p \leq 0.01$, Hb $p = 0.23$, IgM $p = 0.08$, CH50 $p = 0.08$ and ACH50 = 0.63. For all but two of the indicators (lysozyme and osmolality) there was no significant difference at the cage level of the main effects and the decision to combine all cages after 9 months and to pool data of the 4 dietary cages was considered acceptable. It was presumed that the cage effect for lysozyme and osmolality was related to dietary effects rather than husbandry in general and as such the pooling of data would not influence any conclusions on stress under ranching conditions. ANOVA with a conservative decision making probability of $\alpha = 0.01$ again was used to test for differences in indicators between ranching stage groups and post hoc testing of multiple comparisons between means used the Games-Howell procedure. Finally, Spearman's r_s coefficient was calculated for each of the pairwise comparisons of the indicators of individual SBT sampled over the course of the LTH trial. All statistical inference testing was undertaken using SPSS software.

4.3 RESULTS

Cortisol and glucose levels of SBT over the LTH trial are shown in Figure 4.2. Wide variation in cortisol levels again was evident between and within the SBT groups sampled with values ranging from 62.7 $\pm$ 19.6 to 417.8 $\pm$ 84.5 ng/mL.

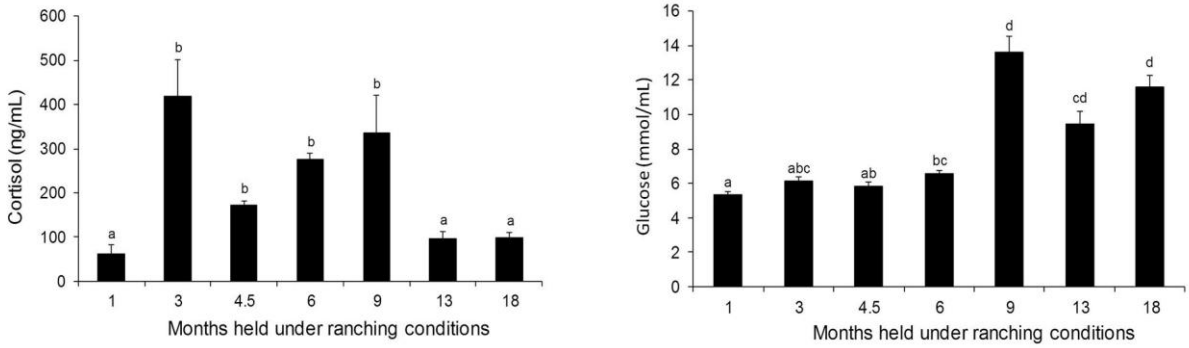


Figure 4.2 Cortisol and glucose levels in SBT (pooled data) for 2005 cohort. Error bars show standard error of mean. Labels group means that do not differ *p=0.01*

There was no statistically significant difference in cortisol levels in the first 9 months of ranching however levels were significantly lower in the second year of captivity at 13 and 18 months after the SBT had been moved into a single cage. Glucose levels ranged from 5.3 $\pm$ 0.2 to 13.6 $\pm$ 0.9 mmol/L and were significantly elevated in the last 9 months of the study (9, 13 and 18 months) compared with the earlier standard ranching timeframes.

Lactate and pH levels are shown in Figure 4.3. Lactate ranged from 2.0 $\pm$ 0.3 to 13.9 $\pm$ 0.9 mmol/L and was significantly elevated above end tow levels (month 1) throughout the LTH trial. Lactate levels at 9 months were higher than any of the previous sampling periods and remained significantly higher than at least two of the previous sampling times (end tow at month 1 and 4.5 months).

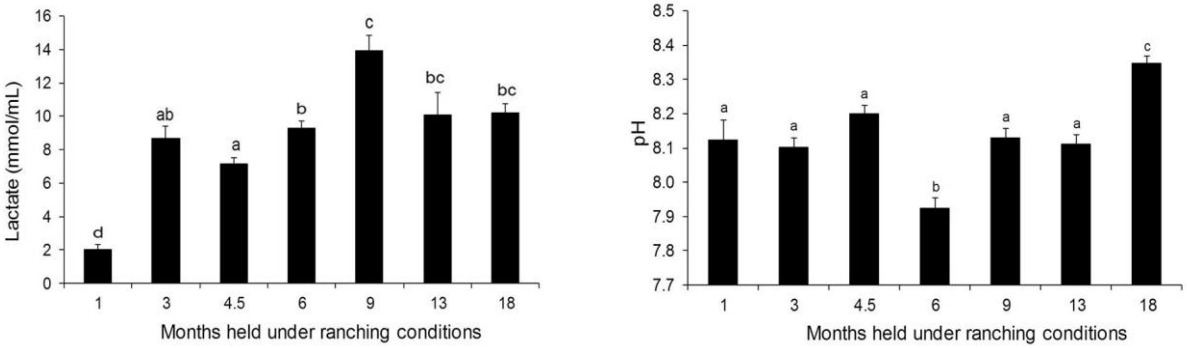


Figure 4.3 Lactate and pH levels in SBT (pooled data) for 2005 cohort. Error bars show standard error of mean. Labels group means that do not differ *p=0.01*

Blood pH ranged from 7.9 $\pm$ 0.03 to 8.3 $\pm$ 0.02 and was not significantly different at most of the time periods sampled. The exceptions were significantly lower pH levels at 6 months and significantly higher pH at 18 months.

Lysozyme and IgM levels are shown in Figure 4.4. Lysozyme levels ranged from 32.1 $\pm$ 2.8 to 85.8 $\pm$ 4.1 µg HEWL/mL and were significantly higher 3 months into the LTH trial than at any other time with the exception of 18 months.

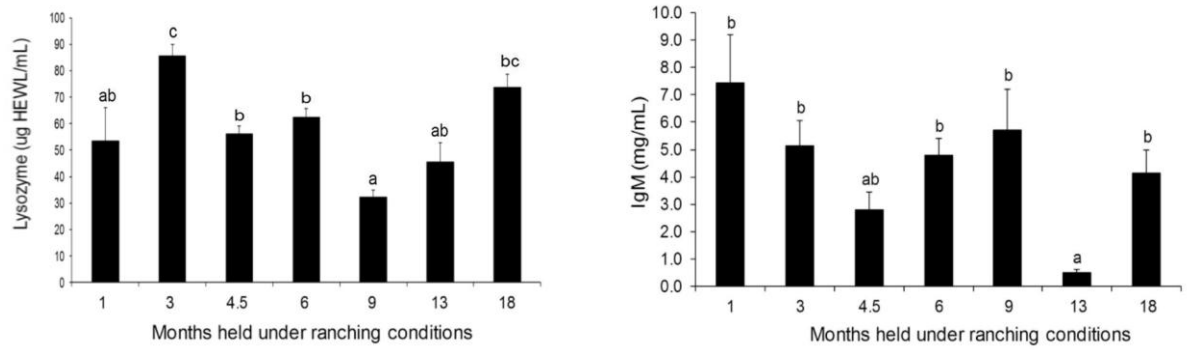


Figure 4.4 Lysozyme and IgM levels in SBT (pooled data) for 2005 cohort. Error bars show standard error of mean. Labels group means that do not differ *p=0.01*

Lysozyme levels were significantly lower at 9 months than the 6 months preceding. There was considerable variation in plasma IgM which ranged from 0.5 ± 0.1 to 7.5 ± 1.7 mg/mL although only one sampling period at 13 months was significantly lower than all other times sampled other than 4.5 months after transfer.

Osmolality and Hb levels are shown in Figure 4.5. Osmolality ranged from 402.6 ± 7.1 to 480.9 ± 11.9 mOsmol/kg and was significantly higher in the last 12 months of the LTH trial (months 6, 9, 13 and 18). Hb levels ranged from 13.8 ± 2.1 to 22.4 ± 0.8 g/dL although there was no significant difference over the course of the trial.

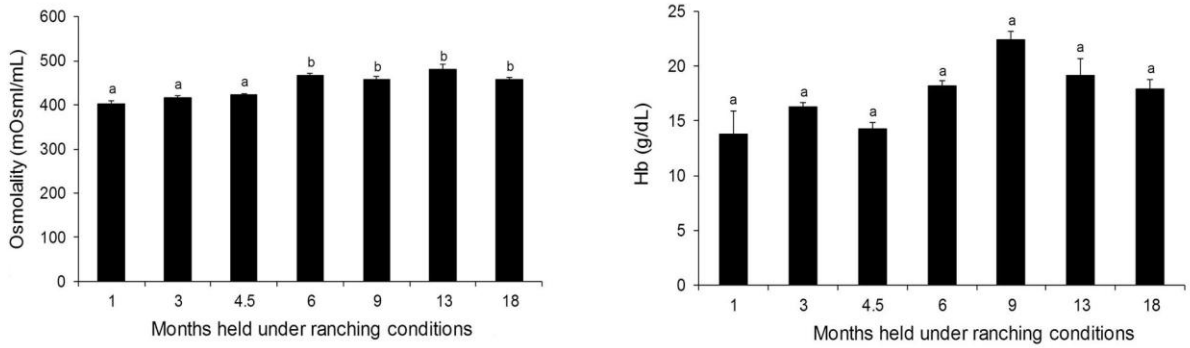


Figure 4.5 Osmolality and Hb levels in SBT (pooled data) for 2005 cohort. Error bars show standard error of mean. Labels group means that do not differ *p=0.01*

Complement activity (CH50) and alternative complement activity (ACH50) are shown in Figure 4.6. Unfortunately laboratory results were only available for the first six months of the LTH trial. Both CH50 and ACH50 were significantly reduced at 6 months.

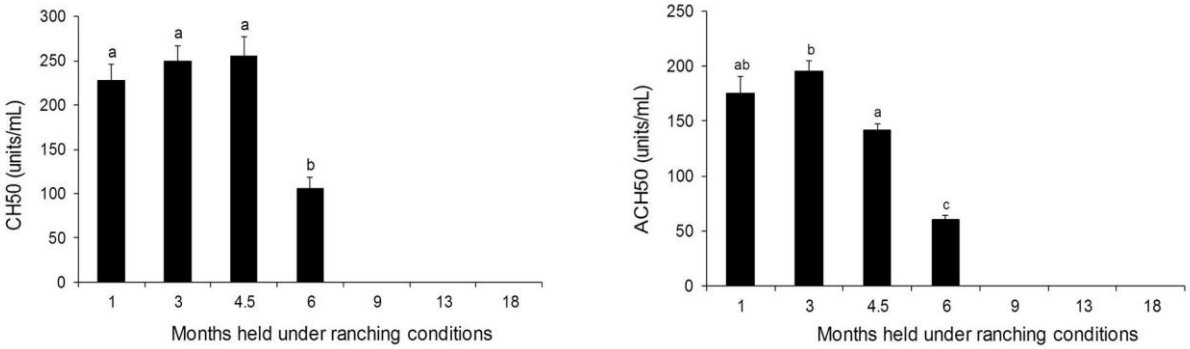


Figure 4.6 CH50 and ACH50 levels in SBT (pooled data) for 2005 cohort. Error bars show standard error of mean. Labels group means that do not differ *p=0.01*

The relationship between individual stress and immune response indicators of SBT over the study period was of interest. Table 3.5 provides a correlation matrix of Spearman's r_s coefficients for the indicators. A significance level of $\alpha = 0.01$ again was adopted and significant associations are highlighted in red.

Table 4.2 Correlation matrix for stress and immune response indicators for LTH trial.
Top value in each cell is Spearman's r_s coefficient, bottom value in each cell is the 2-tailed level of probability. Significant correlations are shown in red. *P=0.01*. Cort = cortisol, Gluc = glucose, Lac = lactate, Lys = lysozyme, IgM = Immunoglobulin M, Osmo = osmolality, Hb = haemoglobin, IGF = Insulin-like growth factor, CH50 = classical complement activity, ACH50 = alternative complement activity.

Cortisol	Cort								
Glucose	-0.012 0.872	Gluc							
Lactate	*0.266* *<0.001*	*0.559* *<0.001*	Lac						
pH	*-0.220* *0.002*	0.165 0.023	-0.170 0.019	pH					
Lysozyme	-0.003 0.969	*-0.236* *0.001*	-0.103 0.161	-0.016 0.826	Lys				
IgM	*0.211* *0.004*	0.005 0.941	0.009 0.903	-0.113 0.115	0.068 0.351	IgM			
Osmolality	0.144 0.047	*0.519* *<0.001*	*0.647* *<0.001*	-0.124 0.082	*-0.235* *0.001*	-0.039 0.589	Osmo		
Hb	0.066 0.406	*0.300* *<0.001*	*0.293* *<0.001*	-0.068 0.390	-0.169 0.035	-0.085 0.284	*0.318* *<0.001*	Hb	
CH50	-0.230 0.011	-0.137 0.132	-0.196 0.030	*0.333* *<0.001*	0.225 0.012	-0.152 0.094	*-0.518* *<0.001*	*-0.247* *0.009*	CH50
ACH50	*-0.263* *0.003*	*-0.241* *0.007*	-0.179 0.047	*0.279* *0.002*	*0.322* *<0.001*	-0.118 0.191	*-0.576* *<0.001*	*-0.251* *0.007*	*0.703* *<0.001*

There were weak positive correlations ($0.20 \leq r_s \leq 0.39$) between cortisol and both lactate and IgM, between Hb and each of glucose, lactate and osmolality, between lysozyme and ACH50 and between pH and both CH50 and ACH50. Weak negative correlations were between cortisol and both pH and ACH50, between glucose and both lysozyme and ACH50, between lysozyme and osmolality and between Hb and both CH50 and ACH50.

There were moderate positive correlations ($0.40 \leq r_s \leq 0.59$) between each of glucose, lactate and osmolality and a moderate negative correlation between osmolality and both CH50 and ACH50. A strong positive correlation was between CH50 and ACH50 ($r_s = 0.703$).

4.4 DISCUSSION

The stress and immune responses of SBT held for longer than the traditional 6 to 8 month cycle of ranching have been detailed above. A similar pattern of primary and secondary stress responses as those measured in ranched SBT (6 to 8 months holding) was found although the duration and extent of responses were higher.

Peak cortisol levels of LTH SBT were notably higher (417.8 $\pm$ 84.5 ng/mL) than the highest recorded cortisol level in commercially ranched SBT (231.5 $\pm$ 40.2 ng/mL). The elevated cortisol levels coincided with the most intensive period of experimentation, immediately after handling and tagging of the SBT and there appeared to be no habituation in the cortisol response in the first 9 months as there was for ranched SBT. Frequent predator interactions were reported during this period of the LTH trial (Ellis et al 2009b) and the elevated cortisol levels over several months likely represent a series of acute stress responses.

Following the transfer of the SBT from experimental cages into a single cage after 9 months of the trial there does appear either a habituation in the cortisol response or an actual attenuation of the eliciting stressors. Cortisol levels returned to the lower level as measured at the trial's start possibly due to a lower frequency of handling, or the greater ease in predator-proofing a single cage compared to protecting several. Cortisol levels at 13 and 18 months of grow-out however remained somewhat elevated above the suggested base reference range of 10 - 70 ng/mL, indicating a modest, chronic stress response of SBT endured during their extended grow-out.

Peak glucose levels of LTH SBT also were notably higher (13.6 $\pm$ 0.9 mmol/L) than the highest recorded glucose level in ranched SBT (8.5 $\pm$ 0.1 mmol/L). As previously shown, glucose levels were influenced minimally under ranching conditions over standard grow-out periods, however they were elevated over the last 9 months of the LTH trial. The peak was 30% higher than the suggested base reference range of 5 to 10 mmol/L and like the elevated cortisol levels indicates a moderate chronic secondary stress response of SBT with their extended grow-out.

Lactate levels in LTH SBT were significantly elevated during the first months of intense handling and sampling in the research cages during dietary manipulations. The peak level of 13.9 $\pm$ 0.9 mmol/L was almost 40% higher than the suggested base reference range of 1 to 10

mmol/L, and remained elevated for the reminder of the LTH trial. The elevated lactate levels further supports the suggested chronic stress response of SBT with extended grow-out. The pH levels of ranched SBT were not considered a reliable indicator of stress and the same was evident for LTH SBT. Notwithstanding a couple of statistical differences, pH levels were always within the suggested base reference range of 7.5 to 8.5 and any changes were not considered to be of physiological significance.

As reported in Section 3.4, stressed SBT encounter greater difficulty in osmoregulating efficiently, and this was the case for LTH SBT. This is a common secondary response in fish in general (Pankhurst 2011) and is partly a function of the role cortisol has in modulating ion exchange processes in the gills (McCormick 2001). It would appear other secondary stress responses may also contribute to osmoregulatory impairment as osmolality continues to be elevated in SBT under both ranching and LTH regimes during habituation of the cortisol primary response.

Lysozyme levels of LTH SBT varied within the range reported for ranched SBT during much of the first 9 months of confinement in smaller cages. Though no clear statistically significant changes were evident, the levels were mostly above the suggested base reference range of 5 to 40 µg/mL, suggesting an enhanced immune response was being mounted by the LTH SBT as a result of holding conditions. IgM levels were not influenced by ranching conditions over 7 months and appeared to be unresponsive to conditions over the longer grow-out period of LTH. IgM levels of the 2005 cohort of LTH SBT were similar to the IgM levels of the next years 2006 cohort described in Section 3.3.1.

Despite the lack of a detectable immune response in IgM levels, the reported pattern of parasitic infection in the LTH SBT suggested other unmeasured cellular immune responses were expressed. The prevalence and intensity of infection by both the blood fluke *Cardicola forsteri* and the gill parasite *Pseudocycnus appendiculatus* was higher in the first six months, and then significantly lower in the subsequent 12 months of LTH (Hayward et al 2008a; Nowak et al 2009b). Seriological evidence indicated a specific antibody response was elicited in the LTH SBT to *Cardicola* infection (Aiken et al 2008) and that the antibody titres remained elevated throughout the trial, in contrast to the lack of elevation in the total IgM. It was suggested that the antibody response was being upregulated by the on-going penetration of the cercaria and that the active response provided protection against juvenile stages so that

recruitment of adult flukes in the heart was minimized (Aiken et al 2008). Cellular immune responses similarly have been reported in other bluefin tuna species.

Infection of ABT by the copepod gill parasite *P. appendiculatus* elicited the induction of the pro-inflammatory cytokines, interleukin-1β (IL-1β), and tumour necrosis factor α (TNF-α), to control the infection (Lepen Pleić et al 2015), whilst constitutive expression of both IL-1β and TNF- α in the gills and skin of PBT indicated a well adapted innate immunity barrier to infectious agents (Mladineo and Block 2010). Environmental stressors such as chronic cold temperature exposure has been shown to upregulate the gene expression of chaperone molecules Hsp70 and oxygen dependent HIF transcripts in PBT (Mladineo and Block 2009) and SBT have displayed similar responsiveness of innate cellular immunity with the induction of the inflammatory mediators TNF-α, IL-1β, IL-8 and cyclooxygenase-2 (Cox2) by LPS stimulation and temperature increases (Polinski et al 2013b). It is likely SBT under LTH would have exhibited a number of cellular immune responses that went unmeasured in this study in response to the stressors induced by extended grow-out and exposure to parasitism.

Though only limited complement (CH50) and alternative complement (ACH50) activity measurement was undertaken in the LTH trial, the last sampling point at 6 months indicated the humoral innate immune response capability of LTH SBT was reduced at that time. A similar drop in ACH50 activity in ranched SBT over the 7 months of grow-out has been reported previously (Kirchhoff et al 2011a, Kirchhoff eta al 2012). In the latter study ACH50 activity dropped from 115.2 $\pm$ 12.3 units/mL 8 weeks into grow-out to 48.2 $\pm$ 4.1 units/mL. Comparable LTH SBT ACH50 activity was 195.3 $\pm$ 9.9 units/mL at 12 weeks into grow-out down to 60.8 $\pm$ 3.7 units/mL at 6 months. A similar drop in ACH50 in a related study was associated with a peak in the abundance of the blood fluke *Cardicola forsteri* (see Kirchhoff et al 2011c), and speculated to have been related to the exhaustion of immune response molecules in reaction to the infection and/or a decrease in host production.

Like those for ranched SBT, the relationships between individual health indicators in LTH SBT indicated that lactate levels rise in association with cortisol increases and that osmoregulatory requirements are increased as the chronic stress ensues. In contrast however, the LTH SBT stress response included a significant glucose response, absent in ranched SBT over 6 to 8 months. The suggested stress axis in LTH SBT is increasing cortisol → increasing glucose → increasing lactate → impaired osmolality. Unlike the ranched SBT sampled in subsequent years (Chapter 3), lysozyme activity of the SBT of the LTH 2005 cohort was

reduced in association with increases in osmolality (r_s = -0.235). The innate immune response, and particularly that of the alternative complement pathway (ACH50) was clearly reduced in association with an increase in cortisol (r_s = -0.235), an increase in glucose (r_s = -0.241) and increase in osmolality (CH50, r_s = -0.518, ACH50, r_s = -0.576).

The LTH trial results supported those obtained for ranched SBT and indicated an enduring stress response over a longer holding period with a suggested reduction in immunocompetence, at least with regard to innate immune response. The strength of the stress response was greater with the increased time in grow-out and included a distinctive increase in glucose response that was not evident within the shorter holding period of commercial ranching. A contributing factor in the displayed stress response, particularly in the first six months, may have been parasitic infection. Other studies have directly implicated parasite infection, and particularly infection by *C. forsteri*, with changes in the stress indicators and humoral immune response of SBT (Kirchhoff et al 2011a, Kirchhoff et al 2011c, Kirchhoff et al 2012).

The results reported in Chapter 3 and this chapter add to the store of knowledge of base levels of stress and immune response indicators of bluefin tuna, and have supported the results of other studies that have documented annual differences in health indicators of wild SBT stock. The reported increased stress response with LTH will assist ranchers in assessing the benefits of extending the captive holding period. Cortisol, lactate, lysozyme and osmolality are of the most predictive value in regards the influence of ranching on the health status of SBT.

In view of the inherent variability in some of the humoral stress responses measured, the melanomacrophages (MMCs) within selected major organs of SBT were investigated as an alternative method of measuring stress (Chapter 5). Histopathological changes in fish are a tertiary stress response and it was speculated that MMC characteristics provided an opportunity to measure an integration of SBT ranching stressors.

CHAPTER 5 MELANOMACROPHAGE CENTRES

5.1 INTRODUCTION

The haemopoietic tissue of fish commonly contains aggregates of pigmented cells termed melanomacrophage centres (MMCs). This terminology has been adopted here following the approach of Roberts (1975) in accepting the broad definition of Edelstein (1971) of melanins including all yellow, yellow-brown and black organic polycyclic polymers of high molecular weight that are insoluble in most solvents and strongly acid resistant. In normal fish MMCs are common in the spleen and kidney, and in some species, the liver (Agius 1979). As noted in Section 1.5, their functions include deposition sites for intracellular bacteria, retention of iron, antigen trapping and presentation to immune cells, and the collection of products of cellular degradation (Wolke 1992, Agius and Roberts, 2003). They accumulate pigments associated with tissue and cellular deterioration ; lipofuscin, haemosiderin and melanin, all of which have the ability to absorb and neutralize free radicals, positively charged ions and other potentially toxic agents (Agius and Agbede 1984, Zuasti et al. 1989). A functional role mirroring that of germinal centres in the humoral immune responses of mammals has been suggested (Ellis 1980) and even a possible evolutionary link (Vigliano et al 2006).

There has been some debate as to the use of MMCs as biomarkers of environmental stress (Section 1.5), although an increasing volume of research over the last decade or so has linked changes in MMC content within organs of fish with sources of stress. Table 5.1 provides examples of the environmental stressors and infectious agents that have been linked to MMC changes in the organs of a variety of freshwater and marine fish. The studies listed investigated changes in the MMCs of fish from wild populations.

In view of their role in immune responses, an increasing focus has been placed on investigating the morphometrics of MMCs in studying the influence of aquaculture and specific husbandry practices on fish health. Montero et al (1999) reported that gilthead seabream (*Sparus aurata*) held at an increased stocking density (from approximately 3 kg/m^3 to 12 kg/m^3) had a significant increase in the percent cover of MMCs in spleen tissue (0.48 $\pm$ 0.27 % up to 0.95 $\pm$ 0.57 %). A group of seabream subjected to a combination of vitamin E diet deficiency and high stocking density had an increased percent cover of MMCs in the kidney tissue (2.86 $\pm$ 0.80 % up to 5.96 $\pm$ 0.88 %).

Table 5.1 Examples of stressors and associated changes in melanomacrophage centres (MMCs) in haemopoietic organs of fish. All MMC changes are increases unless indicated by * to denote a decrease. Unless otherwise cited the source for subsequent species is the same as that for the preceding one.
[2] substantially older wild control fish, [3] Combined with high stocking density, K=Kidney, S=Spleen, L=Liver, PCB=Polychlorinated Biphenol

Stressor	Species	Organ	Source
Fasting	*Scyliorhinus canicula*	S, L	Agius and Roberts 1981
	Salmo gairdneri	K, S	
	Pleuronectes platessa	K, S	
	Xiphophorus helleri	K, S	
	Tilapia zillii	K, S	
	Diplodus annularis	S	Micale and Perdichizzi 1990
	Oncorhynchus masou	K	Mizuno et al 2002
	Paralichthys olivaceus	K	Hur et al 2006
	Hoplias malabaricus	L	Rios et al 2007
Iridovirus	*Trichogaster trichopterus*	K*, S*	Russo and Yanong 2007
Mycobacteria	*Oreochromis niloticus*	S	Manrique et al 2014
Parasites	*Notropis hudsonius*	S	Thilakaratne et al 2007
Pollution			
Cadium	*Tilapai mossambica*	K, S, L	Suresh 2009
Hydrocarbons	*Pseudopleuronectes americanus*	L*	Payne and Fancey 1989
PCB	*Fundulus grandis*	S	Ali et al 2014
	Cynoscion nebulosus	S	
Toxins	*Euryglossa orientalis*	K, S	Taheri et al in press
	Acanthopagrus latus	K, S	
	Cyprinus carpio	L	Fisher et al 2008
Urban	*Rutilus rutilus*	S, L*	Pronina et al 2014
	Notropis hudsonius	S	Thilakaratne et al 2007

Changes in the MMC characteristics of several cultured fish species as a result of dietary manipulations have been reported, sometimes combined with subsequent health challenges. The influence of dietary supplementation and contaminant inclusion has been investigated, as well as exposure to infectious agents. Table 5.2 provides examples of studies undertaken with stocks of cultured fish that have reported changes in MMCs within their organs.

Care must be exercised in interpreting changes in MMCs with the myriad of influences in culture conditions. Wide variations of MMC content in the spleen of Atlantic salmon, *Salmo salar*, occurred within and between vaccination treatments but could not be attributed to treatment effects (Morrison et al 2001). An increased density of splenic MMCs in a study of the inclusion of gramine in the diet of rainbow trout, *Oncorhynchus mykiss*, was considered to be a result of starvation rather than a treatment effect (Glencross et al 2006). In a comparison of farmed versus wild seabass (*Dicentrarchus labrax*) the number of MMCs in the spleen and kidney of farmed fish were reported as being significantly higher by Kurtović et al (2008), yet a later study of the same species comparing wild and farmed fish failed to detect any MMC in the kidney (Saraiva et al 2015).

Table 5.2 Treatments and associated changes in melanomacrophage centres (MMCs) in haemopoietic organs of cultured fish stocks. K=Kidney, S=Spleen, L=Liver

Treatment	Species	Organ	Source
Diet supplementation			
GM soy added	*Salmo salar*	S	Sissener et al 2009
Lactobacillus added	*Lutjanus peru*	S	Reyes-Becerril et al 2012
Uncaria tomentosa added	*Oreochromis niloticus*	S	Yunis-Aguinaga et al 2015
Contaminant inclusions			
Mycotoxin	*Ictalurus puctatus*	K, L	Manning et al 2003
	Oncorhynchus mykiss	L	Woźny et al 2015
Melamine and cyanuric acid	*Lates calcarifer*	K	Phromkunthong et al 2015
Infectious agents			
Myxosporean	*Scophthalmus maximus*	S, K	Sitjà-Bobadilla et al 2006
Monogean gill parasities	*Sparus aurata*	S	De Vico et al 2008
Vibrio	*Pelteobagrus fulvidraco*	S	Zhang et al 2014

Differences in the percent tissue cover of MMCs in the liver of farmed and wild ABT have been reported (Passantino et al 2013).[3] Farmed juvenile ABT from the Zadar region of the Adriatic Sea (3.5 years old) had significantly higher MMC cover in the liver (4.0 $\pm$ 0.7 %) than either farmed juvenile ABT (2.5 years old) from further south in the central Adriatic (1.9 $\pm$ 0.4 %) or farmed adult ABT (5 years old) from the western Mediterranean (1.9 $\pm$ 0.5 %). It was suggested the difference was related to the higher organic pollution in the waters of the Zadar region. MMC numbers in fish organs is known to increase with age (Wolke 1992) and this appears also to be the case with ABT. Wild adults (13 years of age) had significantly higher MMC cover in the liver (5.3 $\pm$ 1.1 %) than the younger farmed adults mentioned above. In contrast, another cohort of farmed ABT that had been in cages for at least 1 year were reported to have few MMCs in liver histology sections and 'scarce' MMCs in kidney and spleen sections (Šimat et al 2009).

Following Schwindt et al (2006), computer image analysis was used in this study to quantify the surface cover of MMC pigments within the tissue of the selected organs rather than the number of MMCs. This avoids problems with defining poorly organized or irregularly shaped macrophage aggregates as often found in some more ancestral species (Agius and Roberts 2003). Though not anticipated to be a problem in the more specialised SBT, this was considered a suitably objective approach to adopt. The aim of this study was to investigate the effects of time under ranching conditions on the MMC surface area cover within selected organs of SBT.

[3] Percentages cited from Passantino et al (2013) are approximate, being transposed from graphical representations of results in μm^2 / mm^2 (10,000 μm^2 / mm^2 = 1%).

5.2 METHODS

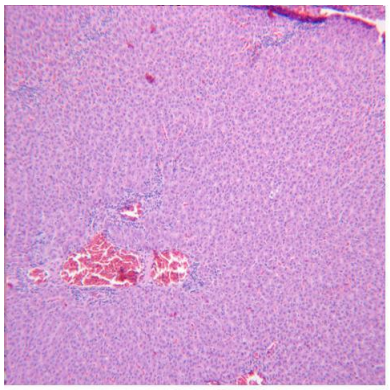
5.2.1 Study design

The study was undertaken over the three year period, 2006 to 2008. A pilot trial in 2006 examined MMCs in the kidney, spleen and liver of SBT in the wild, at the end tow and at harvest. MMCs were observed very infrequently in the liver of SBT as demonstrated in Figure 5.1 and all subsequent analyses were undertaken only with kidney and spleen samples. Note alphanumeric labels associated with the images of histological sections in this chapter are merely identifying sample codes.

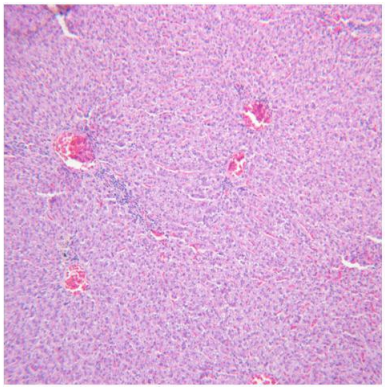
Organ sampling was very much opportunistic at the two earliest ranching stages due to fishing ground remoteness, company confidentiality and availability of experienced sampling personnel. Only one company tow cohort (T1) was available for only sampling periods. An additional mid-season sampling point was added in 2007. The 2008 sampling included a comparison of fish collected from two different cages (denoted 'a' and 'b') at the end of grow-out sourced from the same company's single tow cohort (T1). Table 5.2 summarises the number of fish sampled for each of the groups.

Table 5.2 Sample size *n* for each sample period. T1a and T1b denote fish from two growout cages from the same tow.

Year	Ranch Stage	Kidney	Spleen
2006	Wild	2	5
	End tow	5	5
	Harvest	5	5
2007	Wild T1	4	5
	Wild T2	5	5
	End tow T1	4	5
	End tow T2	5	5
	End tow T3	5	5
	Mid season T1	5	5
	Harvest T1	5	5
	Harvest T3	5	5
2008	Wild T1	5	5
	Wild T2	5	5
	End tow T1	5	5
	Harvest T1a	5	5
	Harvest T1b	5	5



61T21 6LD (Wild)



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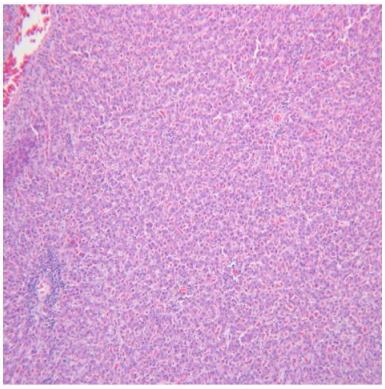
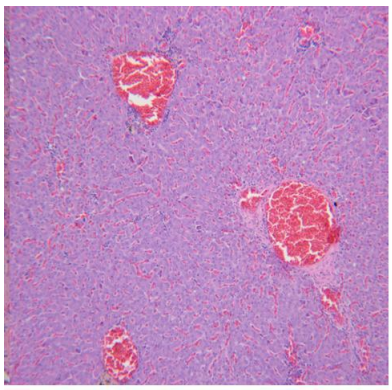
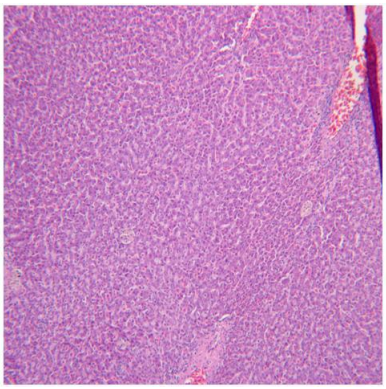


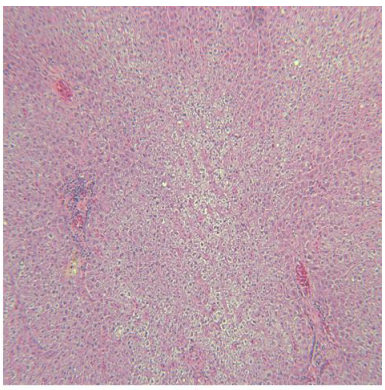
61T21 10LA (Wild)



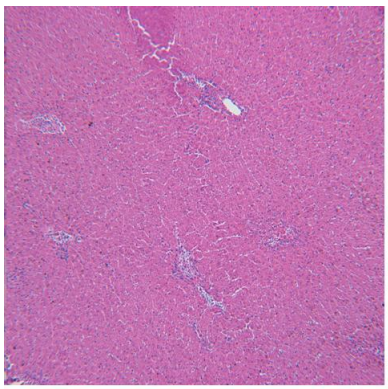
63T21 1LA (End tow)



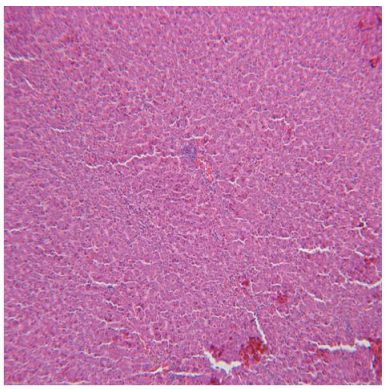
63T21 3LD (End tow)



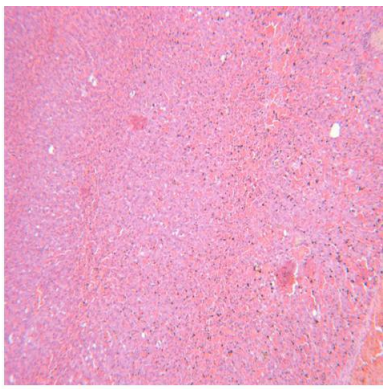
66T40 6LD (Harvest)



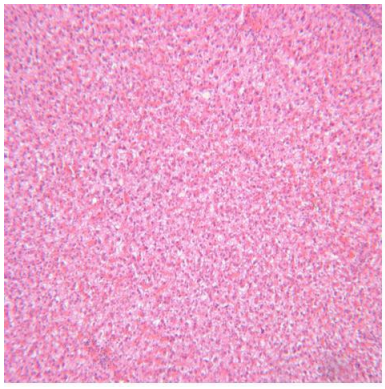
71T21 3LD (Wild)



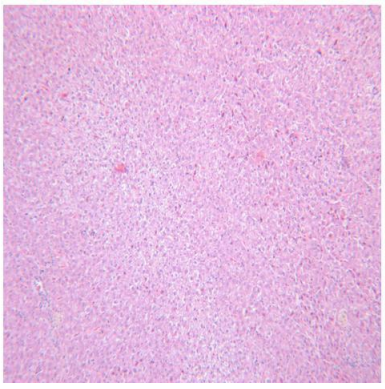
73T21 2LA (End tow)



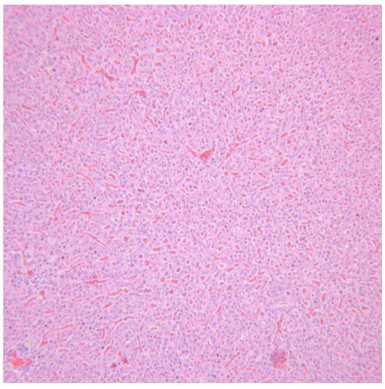
75T19 7LA (Mid season)



78T19 2LA (Harvest)



78T21 36LB (Harvest)



78T21 38LD (Harvest)

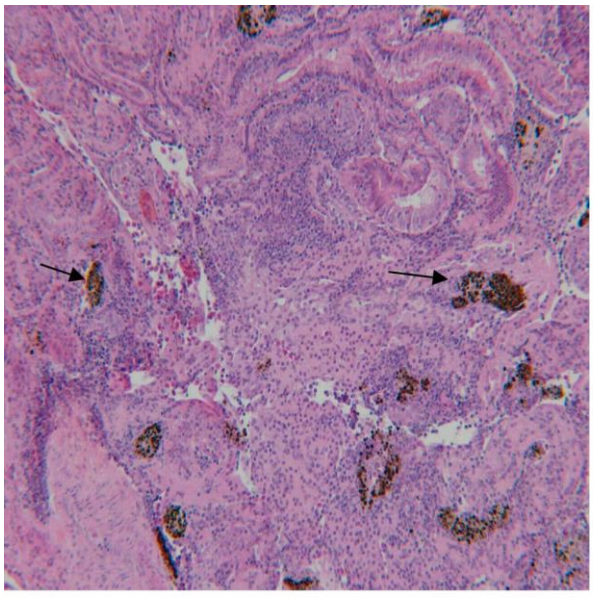
Figure 5.1 Microscope images (x100) of liver histology sections of SBT. Ranching stage given in brackets.

5.2.2 Sample collection and processing

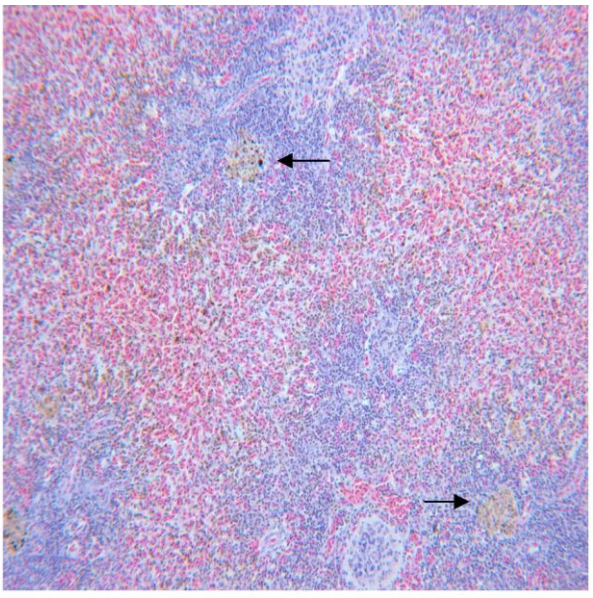
Fish were caught and euthanised as previously described in Section 3.2.1. Gills and viscera were dissected and 4 subsamples of 5 - 7 mm sided cubes taken along the length of the spleen, the head kidney (hereafter simply referred to as kidney) and at least two lobes of the liver. All samples were preserved in 10% buffered formalin for storage before processing. In the laboratory, 1 - 2 mm thick slices from each of the 4 subsamples were set into histology cassettes for subsequent sectioning. Samples were dehydrated through a series of alcohols, cleared in xylene, infiltrated, and embedded into paraffin. Sections (5 μm) were stained with haematoxylin and eosin (H&E). One slide with 4 or more sections was prepared from each organ sampled from each individual SBT.

5.2.3 Image capture and analysis

Observations of histological slides and image capture (x100 magnification) were undertaken with a light microscope (Leica DM1000, Hamburg, Germany) with a camera attachment. Figure 5.2 is an example of a kidney and spleen image with MMCs arrowed. A total of four random images were taken from each slide. The microscope stage and slide section was moved at random in between image capture without use of the eyepieces. The only criteria thus applied in processing an image for subsequent analysis was that at least 50% of the field of view was tissue sample. Repeat images of the same area of tissue were not included.



Kidney slide (87T216 8KA)



Spleen slide (61T21 6SD)

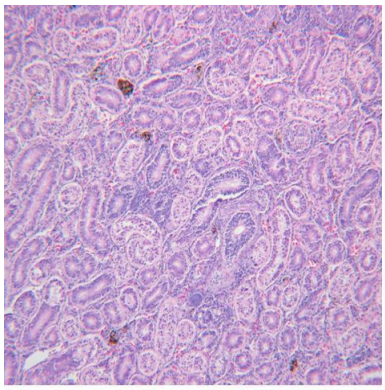
Figure 5.2 Microscope images (x100) of melanomacrophage centres in histology sections of SBT. Arrows point to examples of MMCs

Quantification of the percentage area of MMC coverage of histological sections within the images was achieved with Image Pro Plus 5.0, Media Cybernetics Inc. For kidney sections, the contrast between the MMCs and other tissue parts in the images was enhanced using the Erode Filter prior to quantification. The Measure function and Eyedropper tool was used to select manually all pixels with spectral hues that corresponded to MMCs and change them to the same unique primary colour. The Count/Size function was then used to quantify the proportion of the overall image covered by the selected MMC colour.

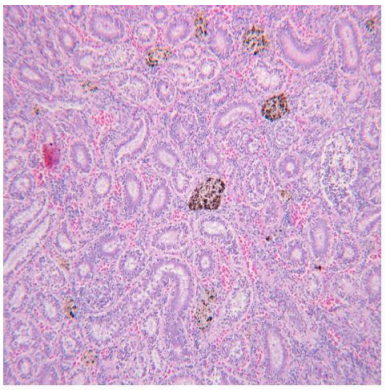
Increasing the contrast within spleen images with the same method used for kidneys proved unreliable. Spleen MMCs were less darkly pigmented than those in the kidney (Figure 5.1 arrowed) and required manual delineation of their outlines with the Lasso tool in Adobe PhotoShop using a stylus and tablet. The delineated areas were then assigned a unique primary colour in Photoshop before being opened in Image Pro Plus for quantification as outlined above for kidney samples. Figures 5.3 and 5.4 provide examples of the range of images of MMCs in kidney and spleen samples respectively.

Using the same image analysis software, Schwindt et al (2006) opted for automated pigment quantification by establishing a composite image mask as a template of colours, gold to brown and black for each organ that the software then used to select pixels automatically in all images. Combined with the splenic contrast consideration mentioned above, such an automated procedure was considered likely to include free pigmented macrophages in the quantification, and hence manual quantification was adopted.

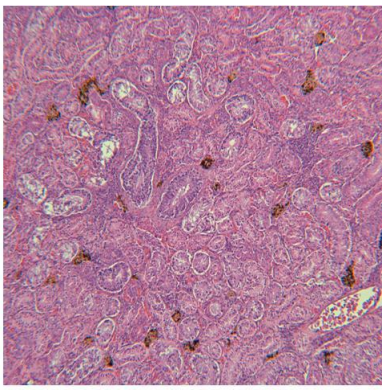
Sample images contained two types of unstained voids which had to be accommodated in the quantification process. The first were those normally associated with the organ structure (ie lumina of blood vessels, ducts, spave between the Bowman's capsules and glomerulus) and as such were included in determining the total area of tissue in the image. The second were sectioning artefacts due to tissue tearing by microtome knives (Figure 5.5a), the sometimes brittle nature of preserved material (particularly spleen, Figure 5.5b) and occasionally from images having been taken that included the extremity of tissue sections on the slide. Schwindt et al (2006) similarly had to accommodate areas of image with no tissue resulting from post-mortem tissue fracture or small organ samples, and did so by removing such void areas from the total area of the image so that final percentages only related MMC pigments to normal tissue.



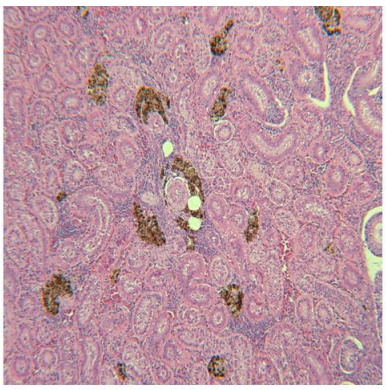
61T21 6KC (Wild)



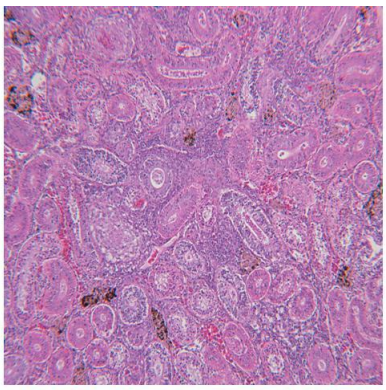
63T21 1KD (End tow)



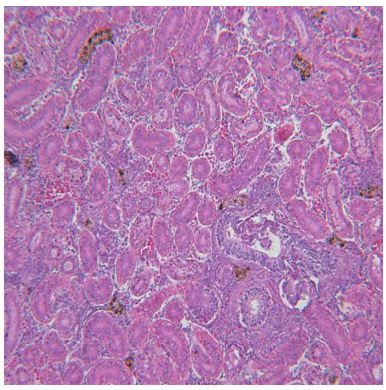
65TT 4KC (Mid season)



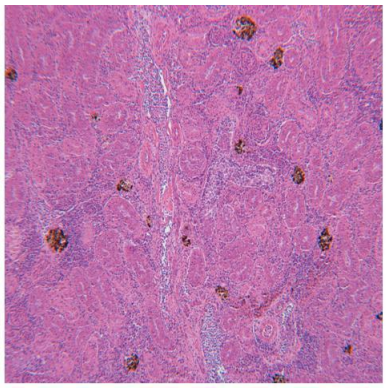
66T40 6KC (Harvest)



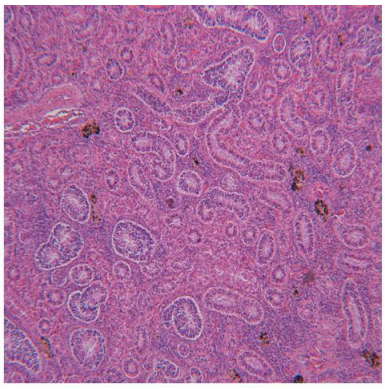
68T214 5KA (Harvest)



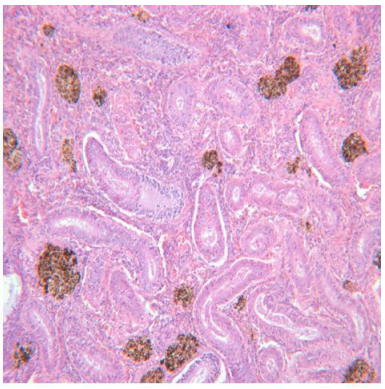
68T214 6KD (Harvest)



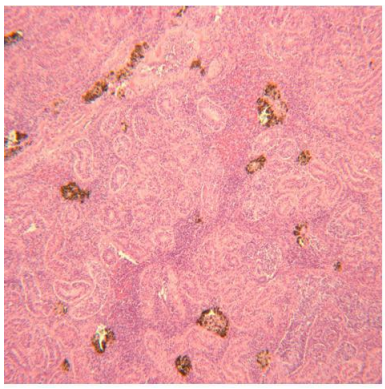
71T21 2KA (Wild)



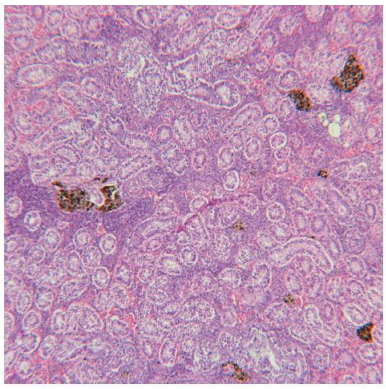
73T21 5KA (End tow)



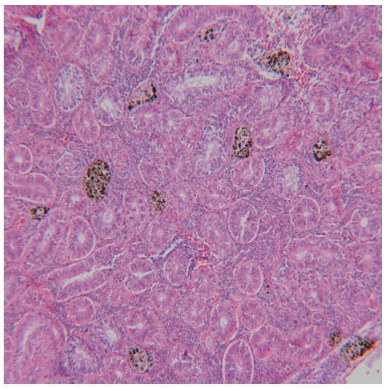
75T19 7KC (Mid season)



81T21 7KD (Wild)

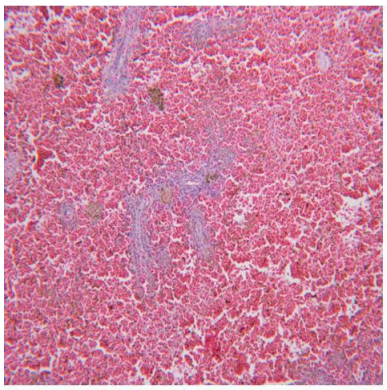


83T21 10KB (End tow)

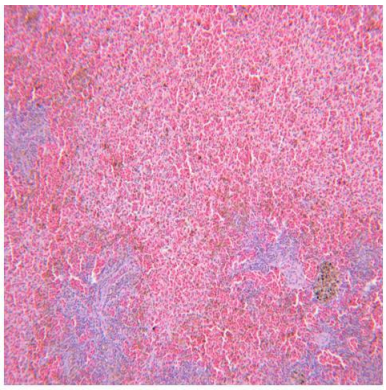


87T213 2KB (Harvest)

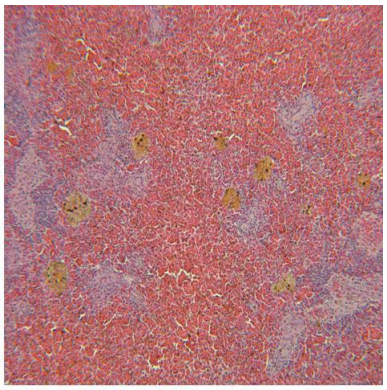
Figure 5.3 Microscope images (x100) of kidney histology sections of SBT. MMCs are very common. Ranching stage given in brackets.



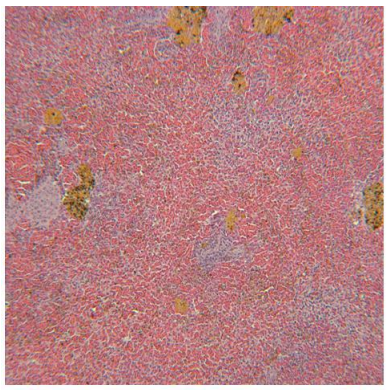
61T21 10SD (Wild)



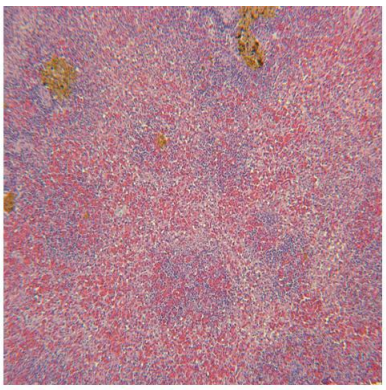
63T21 2SD (End tow)



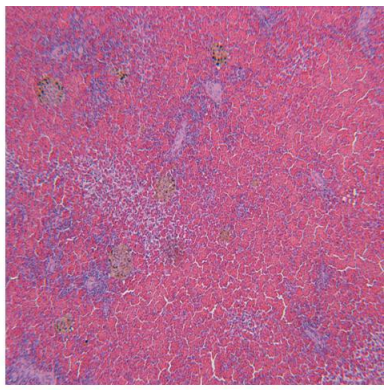
65TT 4SC (Mid season)



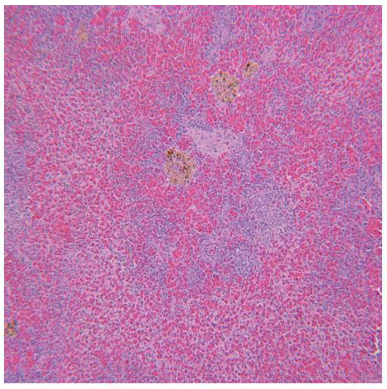
65TT 6SC (Mid season)



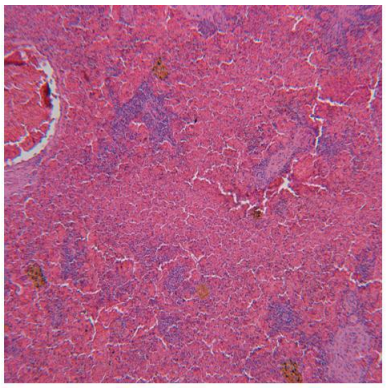
66T40 6SB (Harvest)



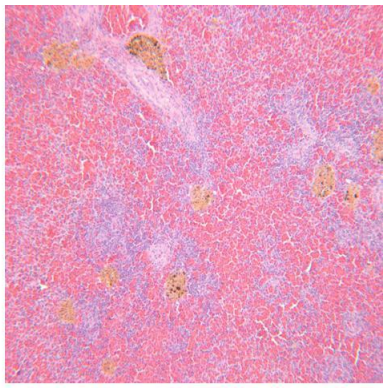
68T214 5SA (Harvest)



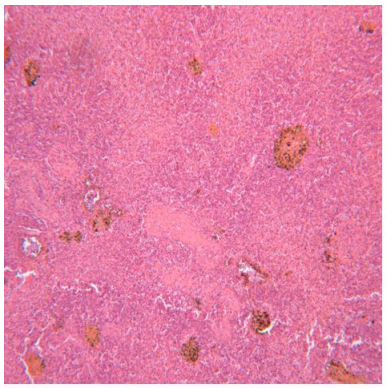
71T22 1SA (Wild)



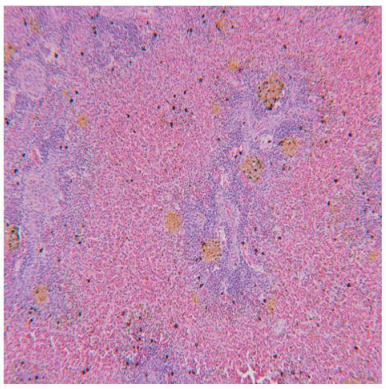
73T21 5SC (End tow)



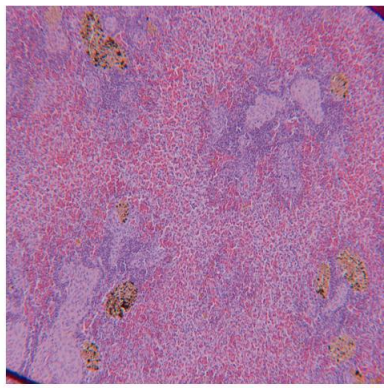
77T21 5SD (Harvest)



81T21 6SC (Wild)



83T21 6SC (End tow)

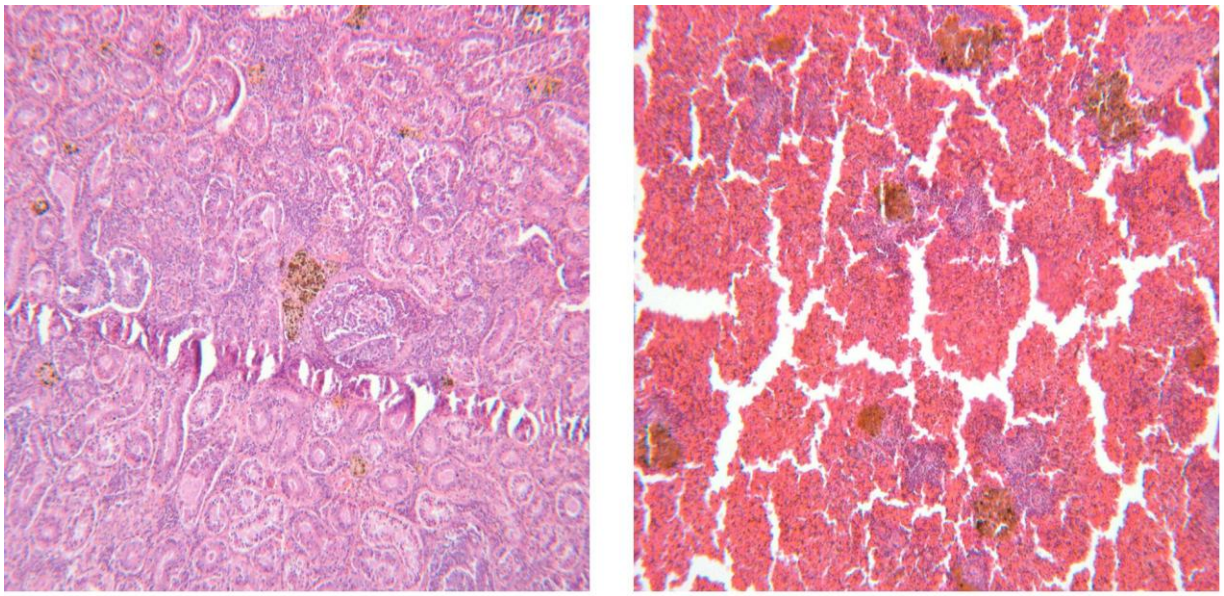


87T213 1SA (Harvest)

Figure 5.4 Microscope images (x100) of spleen histology sections of SBT. MMCs are very common. Ranching stage given in brackets.

In this study voids were excluded from being delineated as either tissue area or MMC area by assigning them a second unique primary colour and quantifying them independently using the Measure→Count/Size functions during image analysis. The MMC primary colour(s) pixels and the void primary colour pixels were quantified individually in relation to the total pixels of the image area. These counts were exported to a spreadsheet and the percentage MMC area calculated as:

$$\text{MMC area (\%)} = \frac{\text{Proportion of MMC colour(s) pixels}}{1 - \text{Proportion of void colour pixels}} \times 100$$



a) Void created in kidney sample by microtome knife (75T21B 4KB)

b) Voids in 'brittle' spleen sample (78T19 4SC)

Figure 5.5 Microscope images (x100) of examples of voids (white areas) in histology sections of SBT.

5.2.4 Statistical inference

The MMC area in respective organs of each SBT sampled was calculated as the mean of the area quantified for each of the four subsamples from the same organ from each individual. Rather than the modified parametric testing methodology adopted for the humoral indicators discussed in Chapter 3, non parametric techniques were used for comparisons of means of MMC area as some analyses involved unbalanced designs due to several missing samples, and the computed metric, percentage of MMC area, was not distributed continuously. Percentages obviously are bounded between 0 and 100. The Kruskal - Wallis analogue of ANOVA was used to test for sample differences in multiple comparisons using a probability level of $\alpha = 0.01$ for statistical significance. Pair wise comparisons between means were undertaken with the Mann - Whitney U test. All statistical inference testing was undertaken using SPSS software.

5.2.5 Pigment differentiation

A series of histochemical stains was used on replicate slides of sections of SBT spleen and kidney tissue in order to identify the composition of the dark brown pigments and granules of the MMC material evident with the H&E stain. For the identification of haemosiderin, a Prussian blue reaction was used which, following processing, stains haemosiderin blue while nuclei are red and the background pink. A Sudan Black B stain was used to identify lipofuscin pigments, which following processing, appear black (together with red blood cells) against a light grey background. Melanin was identified with a combination of the Fontana-Masson silver method which stains melanin and argentaffin granules black (and nuclei red) and a melanin bleach technique which utilises two sections of the same sample and a contrast in visible pigmentation of control and bleached slides. A pigment evident on a control slide that is not present on the bleached slide is melanin. Coupling the melanin bleaching, Fontana-Masson and Sudan Black techniques allows for some differentiation between melanin and lipofuscins.

5.3 RESULTS

The value of tabulating reference base levels of the physiological characteristics of wild SBT has been emphasized. Table 5.3 summarises the percentage of surface area of the kidney and spleen tissue of SBT covered by MMC (hereafter referred to as MMC area) for the five wild cohorts sampled over the course of the study.

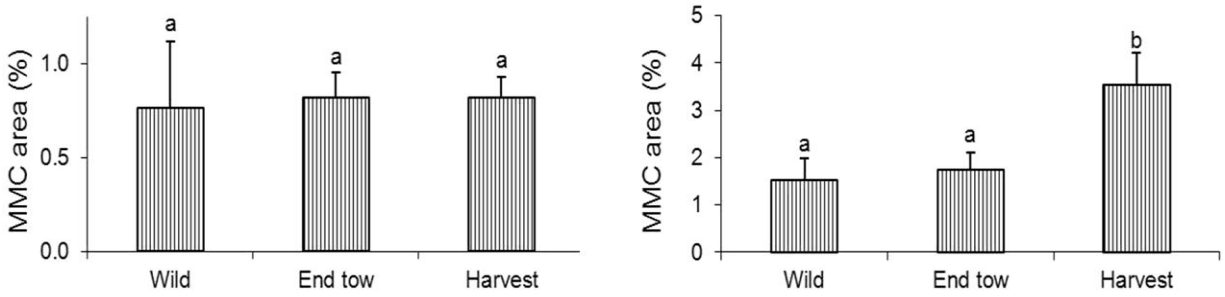
Table 5.3 MMCs in kidney and spleen of wild SBT. Mean $\pm$ standard error of mean. Range in brackets

Year	Cohort	Kidney (%)	Spleen (%)
2006	T1	0.77 $\pm$ 0.38 (0.41 to 1.12)	1.55 $\pm$ 0.45 (0.35 to 2.29)
2007	T1	0.76 $\pm$ 0.10 (0.58 to 1.04)	1.03 $\pm$ 0.15 (0.56 to 1.34)
	T2	0.61 $\pm$ 0.07 (0.47 to 0.83)	1.07 $\pm$ 0.12 (0.82 to 1.52)
2008	T1	2.25 $\pm$ 0.18 (2.04 to 2.95)	1.58 $\pm$ 0.32 (1.06 to 2.75)
	T2	1.30 $\pm$ 0.36 (0.44 to 2.60)	1.04 $\pm$ 0.32 (0.50 to 2.29)

As indicated above, MMCs were observed only very rarely in the liver tissue of SBT and were not quantified in this study. The MMC area recorded in the kidney of wild SBT individuals ranged from 0.41 ± 0.13 % to 2.95 ± 0.26 % over the entire course of the study. MMC area in the spleen of wild SBT varied over a similar range of 0.35 ± 0.09 % to 2.75 ± 0.86 %.

5.3.1 2006 pilot

MMC area in the kidney and spleen of SBT in the 2006 pilot trial is shown in Figure 5.6. Kidney MMC area ranged from 0.77 ± 0.36 % to 0.83 ± 0.13 % and there was no significant difference between the wild, end tow and harvest SBT in 2006. The high variation in the kidney MMC area of wild fish in the pilot trial was due to a reduced sample size (2 SBT, Figure 5.2) as a result of sampling personnel on the fishing ground misidentifying the correct organ for sampling.



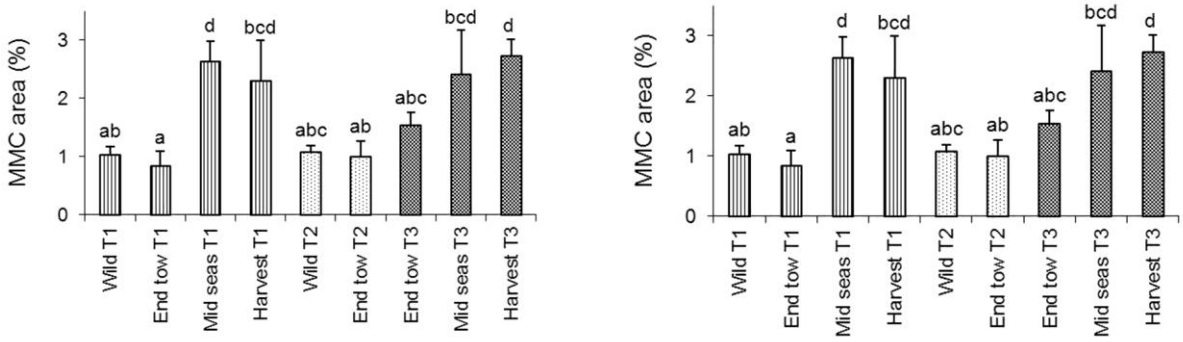
a) Kidney **b) Spleen**

Figure 5.6 MMCs in haemopoietic organs of SBT in 2006. Error bars show standard error of mean. Labels group means that do not differ *p=0.01*

MMC area was higher in the spleen of SBT than in the kidney in 2006, and were significantly elevated at harvest (3.54 ± 0.66%) compared with the low recorded in wild SBT (1.54 ± 0.45%) and also with SBT at end tow (1.74 ± 0.37%).

5.3.2 2007

An extra mid season sampling point was added to the MMC investigation in 2007 (Figure 5.7). The greatest extent of MMC area again was recorded in spleen samples of SBT, with kidney MMC area ranging from 0.59 ± 0.04 % to 2.23 ± 0.96 % and spleen MMC area from 0.84 ± 0.23 % to 2.73 ± 0.28 %. For T1 that had the complete data set there were no statistical differences in kidney MMC areas. However spleen MMC area was significantly higher in mid season T1 SBT than in wild and end tow SBT of T1. Harvest T1 SBT also had significantly higher spleen MMC area than end tow T1 SBT.

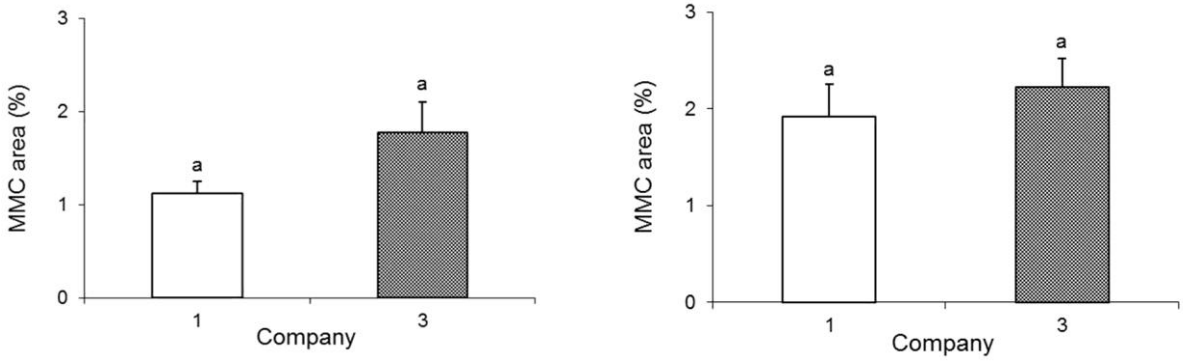


b) Kidney **b) Spleen**

Figure 5.7 MMCs in haemopoietic organs of SBT in 2007. Error bars show standard error of mean. Labels group means that do not differ *p=0.01*

Kidney MMC area of T3 SBT at end tow, mid season and harvest was significantly higher than the wild SBT of the other two tows T1 and T2 which were ranched by a different company. Data from the same ranching stages for T1 and T3 (end tow, mid season and harvest) were pooled and retested to investigate if a difference in the MMC area of SBT ranched by different companies existed. Non parametric 2-way testing using Mann-Whitney U was undertaken for this comparison.

No significant difference existed in the kidney and spleen MMC area of SBT ranched by the two companies (Figure 5.8). As a result, company effects were discounted and 2008 histological sampling focused on comparing two wild SBT groups (T1 and T2) and two harvest SBT groups (T1a and T1b) from the same company.

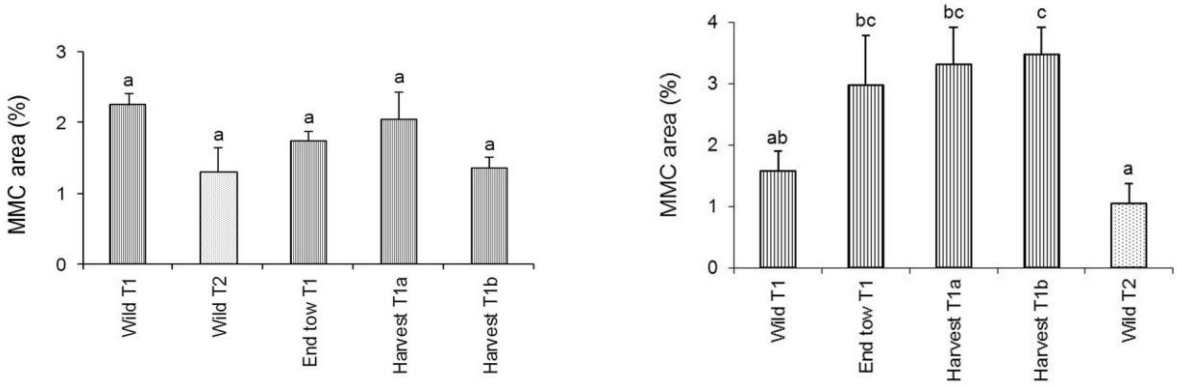


a) Kidney MMC **b) Spleen**

Figure 5.8 MMCs in haemopoietic organs of SBT for pooled data for Companies 1 and 2 in 2007. Error bars show standard error of mean. Labels group means that do not differ *p=0.01*

5.3.3 2008

Kidney MMC area of the five SBT groups sampled in 2008 ranged from 1.37 ± 0.15 % to 2.25 ± 0.18 % and there was no significant difference between groups (Figure 5.9). Spleen MMC area ranged from 1.04 ± 0.32 % to 3.46 ± 0.45 % and considerable variation was evident in the measures within groups. Spleen MMC area at harvest in one grow-out cage T1b was significantly higher than the wild SBT from T1 and T2. SBT sampled from the later ranching stages of the T1 tow all had significantly higher spleen MMC area than the wild T2 SBT group.

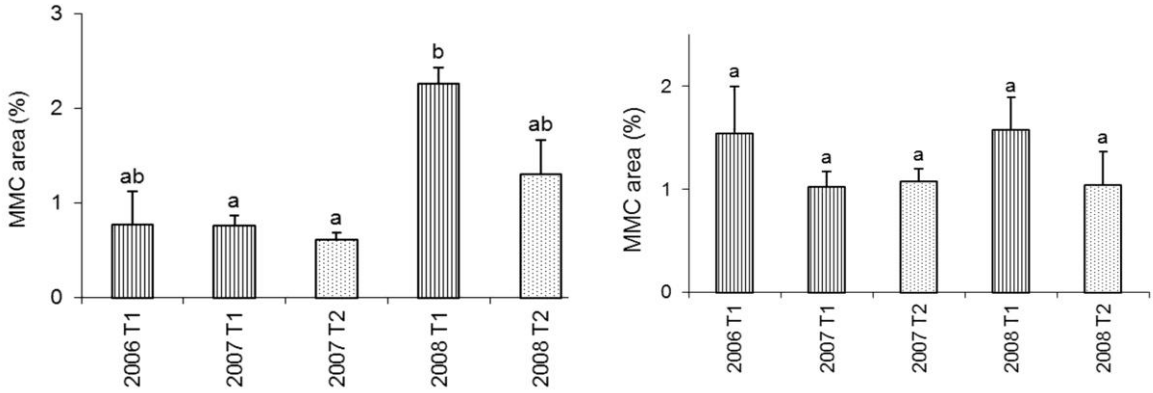


a) Kidney MMC **b) Spleen**

Figure 5.9 MMCs in haemopoietic organs of SBT in 2008. T1a and T1b denote samples from different grow-out cages from the same tow cage T1. Error bars show standard error of mean. Labels group means that do not differ *p=0.01*

5.3.4 Wild SBT

The range of MMC area in the kidney and spleen of wild SBT sampled through the study was provided in Section 5.1. With only one exception, there was no significant difference in the MMC area within the kidney and spleen of wild SBT sampled from 2006 to 2008 (Figure 5.10). A single wild cohort in 2008 had significantly higher MMC area in the kidney than the two wild cohorts from the previous year (2007).

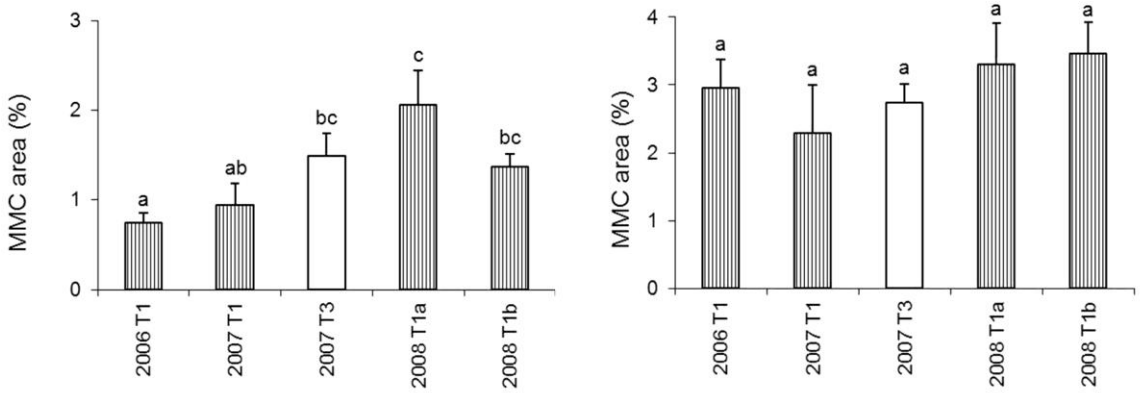


a) Kidney MMC **b) Spleen**

Figure 5.10 MMCs in haemopoietic organs of wild fish for 2006 to 2008. Error bars show standard error of mean. Labels group means that do not differ *p=0.01*

5.3.5 SBT at harvest

The kidney MMC area and spleen MMC area of SBT for the five harvest cohorts sampled are shown in Figure 5.11. A considerable range of 0.74 ± 0.10 % to 2.06 ± 0.38 % was recorded in the kidney MMC area of harvest SBT and the harvest SBT in 2008 had significantly higher kidney MMC area than the harvest SBT from the first years study (2006).



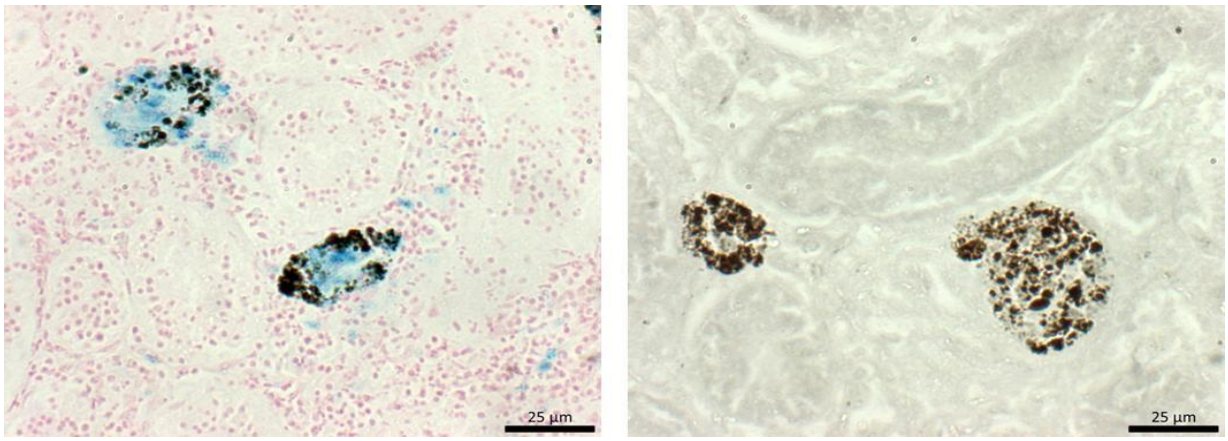
a) Kidney MMC **b) Spleen**

Figure 5.11 MMCs in haemopoietic organs of SBT at harvest 2006 to 2008. Error bars show standard error of mean. Labels group means that do not differ *p=0.01*

One 2008 group (T1a) had significantly higher kidney MMC area than the 2006 T1 group and the 2007 T1 harvest group. Spleen MMC area in harvest cohorts exhibited less variation in range than that of kidney, measuring from 2.30 ± 0.71 % to 3.46 ± 0.45 %. No significant difference was evident in any of the 5 harvest cohorts sampled from 2006 to 2008.

5.3.6 Pigmentation differentiation

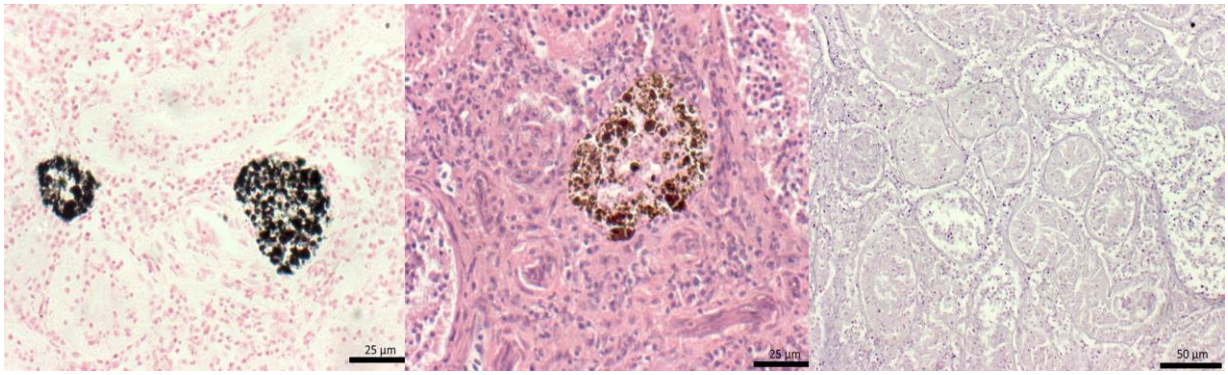
The MMCs of both the kidney and spleen stained positive for all three pigments haemosiderin, lipofuscin and melanin. Figure 5.12 indicates the typical Prussian blue positive response for haemosiderin and the Sudan Black positive response for lipofuscin in kidney MMCs.



Haemosiderin **Lipofuscin**

Figure 5.12 Examples of pigments in MMCs in kidney of SBT. Scale bar is 25 µm

The sequence of steps in the combined Fontana-Masson silver method and melanin bleaching technique is illustrated in Figure 5.13. The Fontana-Masson silver method has stained melanin (and other compounds) black and the comparison of the melanin control and the melanin bleach slides differentiates melanin. Pigment evident in the control but not in the bleached slide is melanin.



Fontana-Masson silver method **Melanin control slide** **Melanin bleach slide**

Figure 5.13 Examples of melanin detection in MMCs in kidney of SBT. Scale bar is 25 µm. (Presented for illustrative purposes only).

Though all pigments were detected in both kidney and spleen MMCs some distinctions were evident subjectively. Visual assessment indicated kidney MMCs were highly positive for lipofuscin and melanin but not as much for haemosiderin. Spleen MMCs were highly positive for Prussian blue indicating high levels of haemosiderin and had a lower level of melanin than kidney MMCs.

5.4 DISCUSSION

The various physiological and immunological roles of MMCs in fish were described briefly in Section 5.1 and reviews have been provided by Wolke (1992) and Agius and Roberts (2003). Very little of this research has involved bluefin tuna. This study appears to be the first into the MMCs of the haemopoietic organs of SBT.

MMCs are most commonly found in the kidney and spleen of most fish and sometimes in the liver (Agius and Roberts 2003). Very rarely were MMCs detected in the liver of SBT in this study. A similar scarcity of MMCs in farmed ABT liver tissue was reported by Šimat et al (2009), although in another study, MMCs were abundant within the liver of ABT (Passantino et al 2013), with ranges from approximately 1.9 % in juvenile farmed ABT to 5.3 % in adult wild ABT. These latter wild ABT were an estimated 13 years of age and much older than the 2

to 4 year old juvenile SBT stocked for ranching. As MMC size and number are known to increase with age (Wolke 1992), it is possible that adult SBT may have more readily detectable MMCs in the liver than the juveniles in this study. Within the juvenile ABT studied, Passantino et al (2013) concluded that one group of farmed juveniles had elevated liver MMC surface area cover as a result of polluted waters in the Zadar region. It is possible that all of the farmed ABT sampled in the Adriatic were subjected to lower water quality than that experienced by SBT in the Great Australian Bight and lower Spencer Gulf and hence had higher/detectable MMC area in the liver. An alternative explanation is that the farmed ABT in the Adriatic had been held captive for a greater length of time than the SBT in this study. Based on the sampling time, stipulated fish size and Croatian fishing seasons of May-June (Mylonas et al 2010), it is likely the farmed ABT studied by Passantino et al (2013) had been held captive for at least one year, 4 to 6 months longer than is usual for SBT. It is possible SBT juveniles held under ranching conditions for more than 6 to 8 months may develop more detectable MMCs in their liver.

Notwithstanding the above qualifications, at least in juvenile SBT under normal ranching conditions, MMCs were not readily detectable in the liver. Further investigations would be required with much older SBT, and probably with SBT held under ranching conditions for a longer period, to test if this distinction holds true for all SBT. Sea bream (*Sparus aurata*) similarly have been reported as having no MMCs detectable in the liver (Montero et al 1999). Pigmented free macrophages, but no MMC aggregates have been reported in the liver of spottail shiners, *Notropis hudsonius* (see Thilakaratne et al 2007) and tilapia, *Oreochromis niloticus* (see Manrique et al 2014), and the latter species only had MMC formations observed in the spleen.

The MMC area in the kidney of wild SBT averaged 1.21 $\pm$ 0.17 % and in the spleen, 1.25 $\pm$ 0.13 %. Though not directly comparable, these levels are similar to the approximately 1.9 % of MMC area in the liver of similarly aged ABT (Passantino et al 2013). A comparison with published percentages of MMC area within the kidney and spleen of other fish is provided in Table 5.4. The reference base levels for MMC surface area cover in the kidney and spleen of wild SBT of about 1.2 to 1.25 % over the three year study period appears conservative in terms of inter-annual change. With only one exception (kidney MMC area in 2008 T1 wild SBT), no significant differences were evident from 2006 to 2008 and it appears the percentage area of MMCs in SBT haemopoietic organs, and particularly in the spleen, may be higher than numerous other fish species. This presumably would provide SBT greater capacity to deal with

the ‘wear and tear’ of the rigors of an active, long-lived life cycle, and increased immuno-competence in coping with parasites and infectious agents.

Table 5.4 Examples of MMC area (%) in kidney, spleen and liver of different fish species. Unless otherwise cited the source for subsequent species is the same as that for the preceding one. Mean ± standard error of mean or a range is given, * approximate values transposed from graphical presentation

Organ	MMC area (%)	Species	Source
Kidney			
	1.21 ± 0.17	*Thunnus maccoyii*	This study
	2.86 ± 0.08	*Sparus aurata*	Montero et al 1999
	0.35*	*Danio rerio*	Capps et al 2004
	1.2*	*Gambusia holbrooki*	
	0.14 ± 0.04	*Paralichthys olivaceus*	Hur et al 2006
	1.06 ± 1.2	*Trichogaster trichopterus*	Russo et al 2007
	0.12 ± 0.14	*Pterophyllum scalare*	
	2*	*Salmo letnica*	Jordanova et al 2012
Spleen			
	1.25 ± 0.13	*Thunnus maccoyii*	This study
	0.48 ± 0.27	*Sparus aurata*	Montero et al 1999
	1.06 ± 1.2	*Trichogaster trichopterus*	Russo et al 2007
	0.12 ± 0.14	*Pterophyllum scalare*	
Liver			
	0.5 to 1.3*	*Cyprinus carpio*	Fisher et al 2008

Following capture, the response of MMCs in the kidney and spleen of SBT under ranching conditions differed markedly. Kidney MMCs did not change significantly with time across the stages of ranching in any of the three years. In contrast spleen MMCs increased in their percentage surface area within the organ in the latter stages of ranching (mid season and harvest). The average spleen MMC area at harvest for the combined three years of the study was 2.95 ± 0.23 %, an increase in area of 2.36 times that of the average splenic MMC area for wild SBT (1.25 ± 0.13 %).

Monterro et al (1999) reported similar results for cultured sea bream (*Sparus aurata*), with increased stocking density alone, dietary manipulation alone and combinations of both, increasing the number of splenic MMCs but not kidney MMCs. The study suggested that splenic MMCs may be more responsive to general stress than kidney MMCs. An increase in splenic MMCs (number and size/area) largely just as a result of holding fish under aquaculture conditions has been reported for sea bass *Dicentrarchus labrax* (see Kurtovic et al 2008), and Atlantic salmon *Salmo salar* (see Sissener et al 2009).

Confirming a definitive cause and effect relationship for the formation and increase in MMC area within SBT organs would require controlled challenge experiments that were beyond the scope of this study. Nonetheless some anecdotal observations can be made within the framework of influences of MMC formation reviewed by Wolke (1992). Increases in MMC number and size in fish with age was unlikely to be a factor in this study. All the SBT sampled were juveniles (2 to 4 years old) and the period under ranching conditions was comparatively brief (7 months). Exposure to anthropogenic pollution sources such as lead or other haemolytic agents during the three year study is very unlikely in the sparsely populated lower Spencer Gulf region, and no obvious point sources of hydrocarbon or other pollutants were evident. Starvation induces degenerative and haemolytic effects that routinely increase MMCs in the organs of fish (Agius and Roberts 1981, Mizuno et al 2002, Rios et al 2007). SBT sampled were held under commercial ranching conditions and fed close to satiation, 6 days per week so lack of feed was considered unlikely.

Quality of feed is another potential cause and increased pigment deposition, particularly of lipofuscin, has been observed in fish displaying nutritional deficiencies (Agius and Roberts 2003, Sissener et al 2009). SBT under ranching conditions are fed a copious volume of feed but of a restricted diversity, the preponderance of which is stored frozen for many months. Studies of storage and feed handling have shown the potential for the loss of vitamins and nucleotides in SBT feed as a result of oxidation/rancidity (Buchanan 2007, Musgrove et al 2007, Musgrove et al 2011) and frozen storage for over a year has increased volatile amine levels in baitfish to the point of making the feed unsuitable for tuna farming (Šimat et al 2009). Lipofuscin is a lipogenic pigment derived from the oxidative polymerization of poly-unsaturated fatty acids (Agius and Roberts 2003) and could be expected to be deposited in greater quantities within the haemopoietic organs of SBT as a result of a suboptimal dietary profile.

Exposure to parasites and infectious agents also has been noted as a cause for increased MMC deposition in fish (Thilakaratne et al 2007) and particularly those agents having a haemolytic effect as haemosiderin is derived from the catabolism of haemoglobin from degenerate red blood cells. Agius and Agbede (1984) reported degenerating red blood cells were commonly observed in the spleen of rainbow trout (*Onchorhynchus mykiss*), plaice (*Pleuronectes platessa*), eels (*Anguilla anguilla*) and tilapia (*Oreochromis niloticus*) and that no degenerating red blood cells or haemosiderin was detected in the kidney of any of these species. Haemosiderin is yellow-brown in colour and found predominantly in the MMCs of the spleen

of fish (Wolke 1992, Agius and Roberts 2002). The lighter yellow-brown colour of the MMCs detected in the spleen of SBT (Figure 5.4) anecdotally suggested that haemosiderin had been increasingly deposited. This was confirmed with the differentiation of pigments which indicated a predominance of haemosiderin in the spleen, and is suggestive of the increased deposition of this pigment during ranching being in response to an infectious agent more so than dietary related.

A range of parasites have been recorded in SBT (Table 1.2), most notably two aporocotylid blood flukes, *Cardicola forsteri* and *C. orientalis*. Aporocotylid infection (*Pearsonellum corventum*) was associated with MMC formation in the heart ventricles of coral trout *Plectropomus leapardus* (see Overstreet and Thulin 1989), and components of fluke egg degeneration were abundant in all of the organs distal to the gills including the liver, spleen and kidney. Some of the MMCs in these organs contained obvious eggs but also degraded products from foreign matter that was no longer recognizable. MMCs in the spleen exhibited a strong positive reaction for the ferric iron, indicating the deposition of haemosiderin (Overstreet and Thulin 1989). In view of the suggested association of *Cardicola* infection with the timing and extent of the stress and immune responses in ranched and LTH SBT (Chapters 3 and 4), and the increased deposition of haemosiderin in MMC aggregates in SBT spleen described here, an obvious potential cause for the increase in splenic MMC area in SBT over the ranching cycle would be infection by *Cardicola* spp.

Given the inter-annual differences reported in the stress and immune responses in Chapter 3, it is interesting how conservative the observed response in splenic MMC area appears to be for ranched SBT. Wild SBT captured in each of the three years had the same splenic MMC area percentage, in each year this percentage increased and there was no significant difference in the splenic MMC area in any of the harvest cohorts across the three years. The observed increase in the percentage surface area cover of MMCs in the spleen of SBT across the 7 month grow-out cycle would appear to be a reliable indicator of stress within the ranching cycle, and possibly of annual *Cardicola* infection. A comparative study of splenic MMC area in praziquantel treated SBT would be of interest to investigate this latter supposition. MMC dynamics appear responsive to at least some stressors within the SBT ranching environment, and further work (in the absence of *Cardicola* impacts) would be required to see if other industry influences on SBT ranching can be monitored in this way.

CHAPTER 6 GENERAL DISCUSSION

A number of general conclusions were evident as a result of this study:

1. Wild SBT populations cannot be considered homogeneous in terms of their health status.
2. Tow methods elicit both primary and secondary acute stress responses in SBT, and if sub-optimal, can influence subsequent survival in the first few months of ranching.
3. Grow-out conditions elicit a modest chronic stress response in SBT that is heightened in duration and extent by longer-term holding to the point of immunosuppression.
4. The SBT stress response of subsequent years ranching cohorts appeared to have increased, particularly post 2007, with an underlying confounding influence being infection by *Cardicola* spp.
5. A tertiary stress response of increased surface area of splenic MMCs is likely related to *Cardicola* infection.

More specific findings were:

a. Shorter tows and lower stocking densities improve subsequent survival in the early months of grow-out. Tow cage stocking densities of less than 0.3 fish/m^3, biomass of less than 5 kg/m^3 and tow speeds of around 0.5 knots are recommended.
b. Within current industry practices, the time taken to transfer fish into grow-out cages has a minimal influence on subsequent mortality rates.
c. Cortisol, lactate and lysozyme levels and osmolality are the indicators most impacted by ranching conditions.
d. There was notable variation in the stress and immune responses of SBT from three different ranching cohorts.
e. Melanomacrophage centres are rarely detected in the livers of ranched SBT.
f. SBT appear to have a high area of MMC cover in their kidney and spleen tissue compared with many other fish.
g. The MMCs of SBT spleen and kidney tissue contain lipofuscin, haemosiderin and melanin, with haemosiderin being predominantly in the spleen and lipofuscin and melanin in the kidney.
h. The pattern of increase in splenic MMCs over the ranching cycle in SBT cohorts from three different years were the same, were more conservative than the inter-annual differences in blood indicators and represent a potential integrating measure of ranching influences on SBT health.

The chronological suite of stress and immune response studies of ranched SBT from the mid 1990s (Watts et al 2002), 2005 (LTH this study), 2006, 2007, 2008 (this study), 2009 Kirchhoff et al 2011b), 2010 (Kirchhoff et al 2011a, Kirchhoff et al 2011c, Kirchhoff et al 2012) to 2012 (Kirchhoff et al 2014), has indicated a devolution in the health status of ranched SBT over that period that was synchronous with increasing mortalities discussed in Section 2.4. An associated inter-annual difference in the health status of wild SBT (Kirchhoff et al 2014), and even differences in health between schools of SBT in the same year (2012), suggested a possible change in environmental conditions that may have played a contributing role during the devolution. Wild SBT in 2008 in this study and 2012 (Kirchhoff et al 2014) had significantly different humoral indicators than other wild cohorts sampled. The nutritional status of fish can influence their stress responses and differential access to prey species is a potential source of variation in wild SBT. Cortisol and glucose levels were elevated with fasting in starved eels *Anguilla anguilla* (see Caruso et al 2010), and a cortisol stress response was maintained for 4 weeks of fasting in sunshine bass, a *Morone* spp. hybrid (Davis and Gaylord 2011).

Within the GAB, seawater temperature and chlorophyll a concentrations, which influence productivity and hence availability of prey, are important determinants of SBT migratory behavior, spatial distribution and residency time (Basson et al 2012). In 2008 when differences in wild SBT health status were reported in this study, sea surface temperatures (SST) in the peak fishing period of February were notably cooler than the preceding and succeeding year (Figure 6.1). In recent years, increases in SST and changes in SBT spatial distribution have been observed (Eveson et al 2014) and annual distribution and environmental differences conceivably could influence access of SBT schools to food sources.

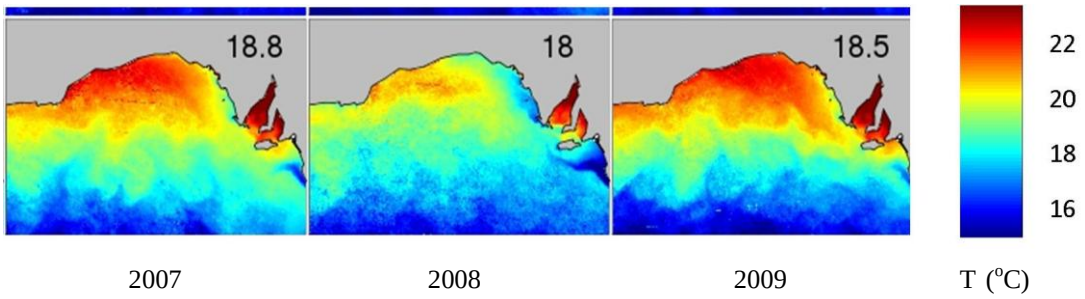


Figure 6.1 Sea surface temperatures in GAB during February for 2007 to 2009. T= temperature Values in each image are mean water temperatures for the area shown.
Source http://www.cmar.csiro.au/gab-forecasts/closeup-historical-sst.html

An anthropogenic source of environmental variation in the GAB that could influence health status either directly, or through disruption to the distribution of SBT, and possibly the access to prey species, is seismic exploration activity. This has been undertaken at varying times over the last 20 years but has increased in frequency in recent years (starting in late 2011) and is currently a research focus for the SBT industry (ASBTIA pers comm). There is scant evidence for direct fish kills from seismic airguns, however behavioural reactions to the noise generated have been documented and can include a startle response, change in swimming pattern and changes in vertical distribution (Worcester 2006). Primary (increase in cortisol) and secondary (increases in glucose and lactate) stress responses have been reported in caged sea bass (*Dicentrarchus labrax*) exposed to seismic air guns up to 2 km away (Santulli et al 1999), with humoral levels reported to have returned to pre-exposure levels by 72 hours after the single transept of the seismic array. Commercial seismic exploration involves months of transepts over extended areas and similar behavioural or physiological responses from schools of SBT (or prey species) to extended seismic exploration conceivably could influence health status of wild SBT in the GAB.

In late 2011 to early 2012 SBT were distributed further to the east than any year since 1990 (Eveson et al 2015) and the commercial fishing season was characterized by dispersed small schools of SBT with flighty surface aggregation behavior (ASBTIA pers comm). Whether natural environmental variation, or disturbance from anthropogenic sources, it is probable that 'patchiness' in the preferred habitat of SBT in the GAB could influence the health status of schools of wild SBT as evident in the stress and immune responses described above.

Through the latter years of the chronology of changes in SBT health status (mid 1990s to 2012), there has been a progressive shift of fishing effort to the east of traditional fishing grounds in response to the eastward shift in the migratory paths and aggregation behaviour of SBT (Eveson et al 2015, ASBTIA pers comm). This eastward relocation by default has resulted in tows of less distance and shorter lengths of time. Cortisol levels have indicated an acute stress response of SBT due to tow conditions. The latency of the cortisol response based on wild SBT sampling methods was suggested to be of the order of 2 to 3 minutes and the base reference level for wild SBT reported (10 to 70 ng/mL) is above that suggested for many free swimming sampled fish species (< 10 ng/mL; Pankhurst 2011). This latter value was obtained from underwater blood sampling of fish by diver and possibly the lower end of the reference level suggested for SBT would be applicable if SBT could be sampled with similar methods.

Shorter tow was a key parameter to optimizing subsequent survival in the early months of grow-out (Chapter 2) along with lower stocking density within the tow cage. There has always been a trade-off between catching suitably sized SBT, in sufficient numbers to fulfill quota, in the time available. Given the restricted fishing season when schools aggregate, increasing the number of tows each operator undertakes to reduce stocking densities is not feasible. A potential solution to minimize the tow stress response would be to increase the number of tow cages each operator has at sea over the fishing season to further reduce overall tow stocking densities. This would mean a commensurate increase in tow cage infrastructure, vessels, crew and at least operating costs (if individual vessels opted to tow multiple cages) and is unlikely to be an option under the present economic conditions of the industry. It has been suggested here that the stress response on tow is attributable partially to restricted feeding during the tow and optimizing volumes of high quality feed would be recommended.

Following the acute cortisol stress response at end tow, some degree of habituation in response occurred. Similar habituation of the cortisol response has been described (Jentoft et al 2015, Kvamme et al 2013, Yang et al 2015) although in SBT an enduring chronic stress response was evident through to harvest. Not surprisingly, another acute primary cortisol stress response was evident after the confinement and handling during harvest.

In ranched SBT the chronic response was somewhat truncated and the stress response axis has been summarized as increasing cortisol → increasing lactate → impaired osmoregulation. The associated increase in osmolality was about 19 % and under the ranching timeframe of 6 to 8 months, IGF levels suggest SBT cope sufficiently with stress to maintain acceptable growth/production capacity. Acute stress has been shown to result in a decrease in IGF-1 in sunshine bass, a *Morone* spp. hybrid (Davis and Peterson 2006) although a general activation of the sympathetic nervous system was suggested rather than a direct cortisol inducement. There was no evidence for a stress related reduction in IGF in ranched SBT.

With increasing time in grow-out, or greater stress exposure, an additional intermediate glucose response was exhibited between the cortisol and lactate responses. In 2006 and 2007 ranched SBT did not exhibit a strong glucose response. Other species similarly have not exhibited the classic increasing cortisol → increasing glucose step in their stress response. Atlantic salmon (*Salmo salar*) exposed to crowding stress exhibited no glucose response at 64 days despite elevated cortisol levels (Basrur et al 2010). Cortisol and glucose levels in coral trout (*Plectropomus leopardus*) subjected to capture and handling stress were variable

although not linked (Frisch and Anderson 2000) and glucose level was not considered a reliable indicator of stress in general in Atlantic cod (*Gadus morhua*; see Perez-Casanova et al 2008) or in cultured pallid sturgeon (*Scaphirhychus albus*; see Nelson and Small 2014). In the latter case it was suggested that the relative energy needs of sturgeon to address acute stress was lower, and it is suggested that the higher metabolic/endothermic capacity of SBT provides for greater modulation of the glycaemic condition of SBT under times of challenge at least in the short term. As the exposure to stress increases the ability to regulate glycaemia appears to be compromised and the elevated glucose response evident in LTH SBT ensues. The elevated glucose response in 2008, and under similar standard ranching timeframes in other more recent studies (Kirchhoff et al 2011b, Kirchhoff et al 2011c) are suggested to be related to the increased influence of parasitic infection (*Cardicola* spp.).

Further evidence for the nature and timing of this interaction was provided in the changing lysozyme responses of the various SBT cohorts studied. In early SBT cohorts (Watts et al 2002), and in the 2005 LTH SBT cohort of this study, lysozyme activity was modestly elevated above base reference levels suggested here (5 to 40 μg/mL). This indicated a response to an increased antigen exposure in captivity and yet lysozyme levels were not a major component of the stress response in the early years of this study. Over the period 2006 to 2008 the lysozyme response became more pronounced during ranching, and continued to be reported as a significant stress response in subsequent studies of SBT to 2010 when it was linked to *Cardicola* infection and the associated mortality peaks from 6 to 11 weeks post transfer. Though acknowledged as a crude metric previously in this thesis, the mortality profile of the industry was shown in Chapter 2 also to have changed over the 2006 to 2008 period. In combination with the stress and immune responses detailed above, the elevated mortality in ranched SBT from an extraneous factor in the mid 2000s (the original premise instigating this study), provides contributing evidence to the influence infection by *Cardicola forsteri* and *C. orientalis* has had on the ranching of SBT over that period.

A possible similar chronology of increased presence of *Cardicola* spp. in tuna farming regions is evident in ABT operations in the Mediterranean. An extensive health survey from 2003 to 2006 of 183 ABT from three Adriatic farms reported 23 species of parasite but no record of *Cardicola* (see Mladineo et al 2008). Recently, the presence of four species of *Cardicola* has been reported in wild and farmed ABT from elsewhere in the Mediterranean (Palacios-Abella et al 2015, Balli et al in press), and it is suggested that the repeated and intensive use of an area for bluefin tuna farming may not only introduce *Cardicola* spp into an area but may pre-

condition the surrounding environment in favour of the parasite, or it's intermediate host. The intermediate host for *Cardicola forsteri* around SBT ranch sites is the terebellid polychaete *Longicarpus modestus* (see Cribb et al 2011) that builds sand tubes in association with shell fragments (unpublished data). Sea cage operations and maintenance practices can increase both the input of organic carbon (Fernandes et al 2007c) and the accumulation of small shell fragments under nets, increasing potential habitat for the terebellid intermediate host.

There are a number of implications for commercial SBT ranching arising from the outcomes of this study and other health investigations. The most obvious already has been implemented, the treatment of SBT within 6 weeks of ranching with the antihelmintic praziquantel to reduce the impacts of *Cardicola* spp. infection (Hardy-Smith et al 2012). This has proved very successful with the peak total industry mortalities of 13.5% in 2010 having reduced to below 1% in the 2015 season (ASBTIA pers comm). Greater attention to net maintenance practices could be given to restrict the available habitat for the fluke's intermediate host. As well as undertaking the investigatory health studies detailed in this thesis, the author has had an adjunct role in determining the life cycle of *C. forsteri* and investigating an appropriate prophylactic treatment with praziquantel (Nowak et al 2010, Cribb et al 2011, Hardy-Smith et al 2012, Appendix 1).

A potential result of the observed health differences within schools of wild SBT is the selection of the most competent schools for capture. Based on this study, the most useful indicator would be lactate concentrations and this conceivably would be possible using a portable lactate meter and non-destructive blood sampling from schools of SBT at sea. Schools of SBT with lactate concentrations less than 2 mmol/L preferentially could be shot in by purse seiners although it is difficult to foresee such an empirical approach being adopted within the weather, operational and competitive constraints at play out on the fishing grounds. Tow speeds of 0.5 to 1 knot were recommended and have been adopted by the company managed by the author. Tow speeds in Croatia of up to 1.2 knots were reported by Katavic (2003b) and the recommendation from this study would be to reduce such speeds.

Innate immune activity (CH50 and ACH50) was affected little over the initial months of ranching but was decreased significantly after 6 months, indicating immunosuppression would be a likely obstacle to extended grow-out periods. The generic immune activity of IgM appears unresponsive to stress over at least standard ranching timetables suggesting at lease some base immunocompetence that would assist SBT in meeting challenges from new antigens and

infectious agents. A specific anti-*Cardicola* antibody response has been well documented in SBT (Aiken et al 2008, Kirchhoff et al 2012) and a similar cellular immune response is mounted in PBT following first infection by *Cardicola orientalis* (see Polinski et al 2014). It is likely that first infected SBT are impacted most and that subsequent reinfections are fought by this adaptive immune response.

Interestingly, earlier research of the innate immune response of ranched SBT from the mid 1990s reported the opposite finding, with ACH50 (and CH50) activity increasing from around 5 units/mL at 10 weeks up to 42 to 50 units/mL after 7 months (Watts et al 2002). The increase was concluded to result from an increased exposure to antigens as SBT were held longer in cages, and the authors raised the possibility of the increased immune response not being sustainable. This suggests the stress profile under ranching conditions has increased over the last 15 years and the unsustainability of an increased innate immune response was a reality in the mid to late 2000s. Initial research on dietary immunostimulants indicated that lysozyme activity was enhanced by vitamin (E and C) supplementation but other stress and immune indicators measured including cortisol, lactate, glucose, pH, ACH50, and performance measures such as survival, condition index and crude fat were not improved commonly through dietary supplementation (Kirchhoff et al 2011b). In view of the LTH immunosuppression, a successful regime of immunostimulation is considered essential to hold SBT successfully for an extended grow-out period.

Melanomacrophages (MMCs) play an important role in the immune system of fish (Agius and Roberts 2003) and the capacity of SBT to meet the challenge of antigenic challenge would appear enhanced by the comparatively high surface area coverage of MMCs in their haemopoietic organs relative to other fish species. It has been suggested here the additional MMC area is linked inherently to the high wear and tear processes encumbered up on a high order, apex predator. Splenic MMCs in particular increase in surface area over the ranching cycle, due to a suggested combination of initial restricted feed volumes on tow, sub-optimal feed quality during grow-out and increased exposure to parasitic infection. The response in changes of splenic MMC cover area appear more conservative in regards consistency in timing and extent than the changes reported in the other stress and immune responses measured, and would provide a reliable, albeit post-hoc, quantification of at least *Cardicola* spp. infection effects, and possibly of different ranching practices for comparative purposes.

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APPENDIX 1 ASSOCIATED RESEARCH PUBLICATIONS

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